

ABSTRACT

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INCORPORATING GRAPE SEED
ANTIOXIDANTS INTO A FUNCTIONAL
FOOD MODEL

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Consumption of foods rich in natural antioxidants may potentially reduce the risk of chronic illnesses. This study examined feasibility and consumer acceptability of creating a functional food rich in natural antioxidants from cold-pressed grape seed oil and flour. The first study investigated and compared five grape seed varieties, and found Chardonnay grape seeds contained the highest quantity of health-beneficial properties. Consequently, addition of Chardonnay grape seed flour and oil to bread significantly increased health-beneficial properties. Baking conditions influenced antioxidant properties of bread, indicating processing conditions may affect antioxidant activity in finished food products. In addition,

the consumer sensory evaluation study found control bread was preferred over bread containing grape seed flour and oil; however, grape seed containing bread had an overall positive reception. Incorporation of grape seeds into bread may be effective in incorporating health-beneficial compounds into the diet; however, further studies on long term health effects should be conducted.

INCORPORATING GRAPE SEED ANTIOXIDANTS INTO A FUNCTIONAL
FOOD MODEL

By

Team Innovative Medicines for Maladies Utilizing Nutraceutical Enhancements
(IMMUNE)

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Chapter 1: Introduction

Introduction

Chronic diseases, such as heart disease, cancer, obesity, and diabetes, are currently the prominent cause of death in the United States. The Centers for Disease Control and Prevention (CDC) estimates that 7 out of 10 deaths among Americans each year are from chronic diseases (CDC, 2010). These long-term illnesses carry a substantial financial burden. According to the CDC, they are responsible for three dollars out of every four dollars that is spent on healthcare in the United States (CDC, 2005). The impact of these types of illnesses is not restricted to developed nations. The World Health Organization (WHO) reports that chronic illnesses cause roughly 60% of all deaths globally (CDC, 2011). Significant research has been devoted to finding novel methods for the prevention and treatment of chronic disease.

In 1992, research sought to identify the relatively low incidence of chronic diseases in France as opposed to in the United States despite the French diet being shown to be similar to American diet rich in saturated fats typically associated with promotion of cardiovascular disease. This phenomenon was termed the French Paradox (de Lange, 2007). The French Paradox led to increased research regarding the differences between the diets and lifestyles of the French and American people; the most striking distinction was the fact that the French were accustomed to consuming wine daily (Renaud & de Lorgeril, 1992). More recent studies have shown that chemical compounds found in grapes and wine, such as resveratrol, offered a possible explanation to the French Paradox (Dulak, 2005), and reinforced the importance of diet in the

prevention of chronic disease. Resveratrol is just one of many polyphenolic antioxidants found in grapes and grape seeds and will be discussed in further detail below.

Genetic predisposition and lifestyle/environment choices are the two main risk factors for chronic illnesses. Since innate genetic traits cannot be altered, efforts toward the prevention of long-term illness focus on improving lifestyles. Risks for developing chronic illnesses has been linked to oxidative stress, which has multiple causes, including overexposure to sunlight, smoking, daily exposure to certain chemicals, and improper diet. The human body functions through carefully monitored oxidation-reduction reactions, both inside and outside the cells. Oxidative stress can shift this delicate balance, resulting in an elevated level of free radicals in the body (Hennig *et al.*, 2007).

Free radicals are very reactive chemical species that exist in the human body as a natural product of metabolism and function. Sustained elevated levels of oxygen centered free radicals experienced during oxidative stress can lead to uncontrolled and undesired reactions that damage the macromolecules in cells, including proteins, lipids, and DNA. Damage to these molecules can result in an increased risk of cancer and cardiovascular disease (Seifried *et al.*, 2007).

Dietary choices play an important role in disease prevention. It is known that certain fruits and vegetables contain phytochemicals, essential fatty acids, traditional vitamins and minerals, and other bioactive compounds. However, the CDC released a report in 2005 stating that only 33% of American adults eat fruit two or more times daily and only 27% eat vegetables three or more times daily (Blanck *et al.*, 2005). The phytochemicals of fruits and vegetables have been shown to reduce the risk of cancer and cardiovascular disease (Liu, 2003).

There is growing interest in the health enhancing role of certain foods and food components. This has led to the development and use of the term “functional foods.” According

to the Institute of Food Technologists (IFT), a “functional food” is considered a food that provides an additional physiological benefit beyond that of its inherent nutritional value (Bidlack *et al.*, 2005). Functional foods can be specifically designed to incorporate antioxidants, which are chemicals with the ability to scavenge free radicals and reduce oxidative stress in the body (Eastwood, 1999). As opposed to pharmaceuticals, they also have the potential to be produced economically and made accessible to a large number of people.

Grape seeds can be exploited for functional food development, as they contribute health beneficial properties and are a byproduct of the juice and wine-making industries. Most antioxidants found in grape seed extracts are polyphenolics (Shi *et al.*, 2003). The key types of phenolics present in grapes and grape seeds are proanthocyanidins, anthocyanins, and resveratrol (Bozan *et al.*, 2008; Lafka *et al.*, 2007; Yilmaz & Toledo, 2004). Studies have shown that proanthocyanidins have preventative effects against cancer, heart disease, and aging by inhibiting malignant cell growth, delaying heart cell death, and maintaining membrane integrity as cells age (Prior & Gu, 2005; Karthikeyan *et al.*, 2007; Nandakumar *et al.*, 2008).

Anthocyanins are the pigments which give grapes and grape seeds their red and purple color. Anthocyanins have been reported to exhibit health-promoting properties, including anti-inflammation of blood vessels and reduction of platelet coagulability, which may reduce risks of developing atherosclerosis, an initial step in cardiovascular disease (Mazza, 2007). Studies have shown that resveratrol, as well as anthocyanins, may positively affect heart health by modulating proteins regulating immune responses, including inflammation of the vascular system (Falchetti *et al.*, 2001; Tao *et al.*, 2004). Overall, polyphenolics appear to be potentially capable of preventing cancer and cardiovascular diseases.

In addition to polyphenolics, antioxidants found in grape seeds include certain vitamins, specifically vitamin C (ascorbic acid) and vitamin E (tocopherols). Vitamins C and E, especially when tested in combination, have been shown to positively affect the health of those with heart disease and diabetes. Vitamin E has been reported to increase the efficiency of cholesterol scavenging by monocytes. This property may be linked to the prevention of atherosclerosis, or the accumulation of plaque lining blood vessel walls, that causes many cardiovascular health problems (Cachia *et al.*, 1998). Also, treatments utilizing vitamin C and E supplements alleviated the symptoms of Type II diabetes patients (Chui & Greenwood, 2008).

Grape seeds are also a source of healthy fatty acids and dietary fibers (Cao & Ito, 2003). Some unsaturated fatty acids contained in grape seed oil, such as α -linolenic acid (ω -3) and γ -linolenic acid (ω -6), are considered essential fatty acids because they cannot be produced by humans (Smith, 2007). Consumption of unsaturated fatty acids has been correlated to a reduction of cardiovascular disease, cancer, hypertension, and autoimmune disorders (Aronson *et al.*, 2001). Additionally, dietary fiber makes up a considerable portion of grape seeds and peels—about 80% of their dry weight—and has been linked with lower risks of heart disease, obesity, diabetes, and colon cancer (Goñi *et al.*, 2005). The addition of grape seeds to a staple food may increase the quantity of fiber in the diet.

Grape seeds are produced en masse as a byproduct from wine and juice production industries. However, their disposal has been shown to negatively impact to the environment. Grape seed waste has high concentrations of organic (carbon-rich) substances. The decomposition of this material decreases the amount of oxygen available to other organisms, resulting in detrimental effects on the ecosystem where they are disposed (Lafka *et al.*, 2007). Therefore, if grape seeds were adopted as a functional food ingredient on a commercial scale, the

potential exists to raise the agricultural value of grapes and significantly decrease the amount of waste produced by the wine and juice industries, minimizing the negative environmental impacts of grape processing.

The purpose of Team IMMUNE's (Innovative Medicines for Maladies Utilizing Nutraceutical Enhancements) project is to increase the intake of health-beneficial compounds in the American diet without dietary supplements. We explored the feasibility of incorporating the antioxidant properties of grape seed by-products from the wine and juice industry into bread, a staple food in the American diet, in accordance with the IFT definition of a functional food. Specifically, cold-pressed (temperature controlled processing method) grape seed flours and oils were used. The ultimate impact of our project could provide a convenient avenue for reducing medical costs incurred by chronic illnesses and introduce a new use for otherwise wasteful by-products of a large industry. Our study has the added benefit of increasing economic value of grape seeds, which are currently disposed of as a waste product of wine and juice production.

Our project had three main research objectives. First, we measured the health-beneficial properties of five varieties of cold-pressed grape seed flours and oils (Chardonnay, Concord, Norton, Ruby Red, and White) to determine which one had the highest potential for development of a model functional food. We then incorporated the seed flour and oil of this variety, Chardonnay, into a bread product and determined the optimal baking temperature, time, and flour composition necessary to produce a viable final bread product that retains the most health-beneficial properties. Finally, we conducted an institutional review board (IRB) approved sensory analysis of our product on freshman Gemstone students at the University of Maryland College Park Campus.

Chapter 2: Antioxidant Properties and Phenolic Components of Grape Seeds

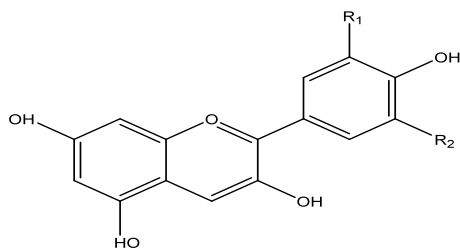
Introduction

Grapes are one of the largest fruit crops in the world, with approximately 66 million tons produced worldwide in 2007, and over 6.1 million tons produced in the United States alone (FAOSTAT, 2007). Approximately 86.6% of fresh grapes are processed to produce wine, jams, and grape juice (Maier *et al.*, 2009). Seeds comprise 5% by-mass of grapes and are a primary by-product from grape processing industries. Grape seeds are composed of 10-20% oil, along with fiber, protein, and other components, including phenolic antioxidants (Kim *et al.*, 2006; Choi & Lee 2008). Investigation into the health beneficial properties such as antioxidative capacity is important for development of value-added utilization of grape seeds (Parry *et al.*, 2006). A number of studies have investigated antioxidant components of grape seeds and seed fractions (Luther *et al.*, 2007). Antioxidant properties of grape seeds, seed fractions, and individual grape antioxidant compounds have also been evaluated. This review summarizes the available information on phenolic components in grape seeds, their antioxidant properties, effects of post-harvest treatments on grape antioxidants, and analytical considerations for grape antioxidant property estimation.

Phenolic Components

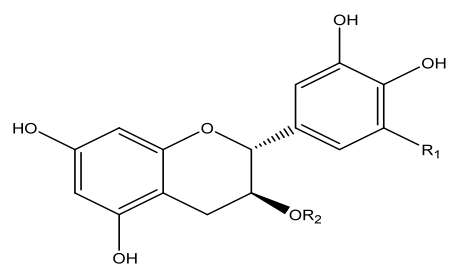
Total Phenolic Content

Grape seeds are rich in phenolic compounds such as anthocyanins, the glucosides of anthocyanidins (**Fig. 2.1A**), catechin (**Fig. 2.1B**), and gallic acid (**Fig. 2.1C**).

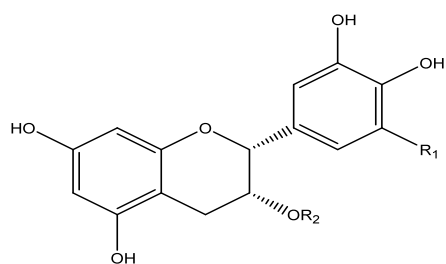


Anthocyanidin	R1	R2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OMe	OMe
Pelagonidin	H	H
Petunidin	OMe	OH
Peonidin	OMe	H

Figure 2.1A



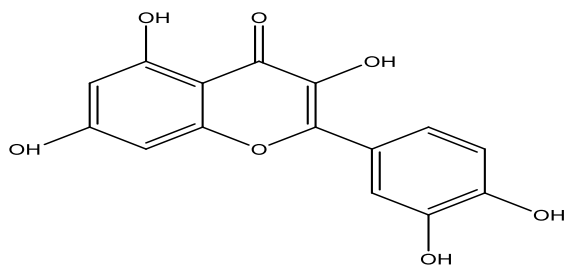
Catechin



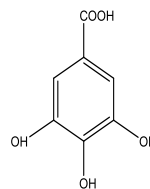
Epicatechin

Catechin	Epicatechin	R1	R2
(+)-Catechin	(-)-Epicatechin	H	H
(+)-Gallocatechin	(-)-Epigallocatechin	OH	H
(+)-Catechin-3-gallate	(-)-Epicatechin-3-gallate	H	Gallate
(+)-Gallocatechin-3-gallate	(-)-Epigallocatechin-3-gallate	OH	Gallate

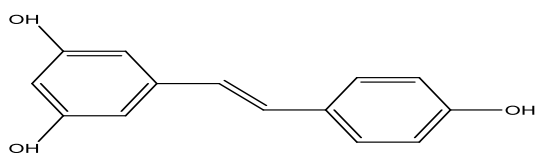
Figure 2.1B



Quercetin



Gallic Acid



trans-Resveratrol

Figure 2.1C

Figure 2. 1

Chemical structures of grape phenolic compounds. (A) Anthocyanidins; (B) Catechins (structure on the left is a (+)-catechin skeleton, while the structure on the right is an (-)-epicatechin skeleton); and (C) Quercetin, gallic acid, and *trans*-resveratrol.

Polyphenolics generally contain two or more hydroxyl groups attached to a conjugated ring system such as a benzene ring. Polyphenols contribute to the overall antioxidant properties of grapes and may have important health benefits, including possible preventative effects against cancer (Fan & Lou, 2004) and cardiovascular diseases (Zern *et al.*, 2003; Zern *et al.*, 2005). Anthocyanins have been shown to protect against lipid peroxidation and DNA damage in rat hepatoma cells *in vitro*, demonstrating potential anticarcinogenic properties (Lazzé *et al.*, 2003). Flavanols, such as catechin, have demonstrated inhibitory effects on platelet reactivity *in vitro*, a property which may reduce the risk of cardiovascular disease (Pearson *et al.*, 2005). Gallic acid has been shown to exhibit selective cytotoxicity *in vivo* against a variety of human and mouse cancer cells (Inoue *et al.*, 1995). Additionally, grape seed extracts rich in polyphenolics have been found to reduce biomarkers of Alzheimer's in a rat model (Thomas *et al.*, 2009). This result was supported by a separate finding suggesting that catechins from grape seed extracts fed in a controlled diet to rats were able to cross the blood-brain barrier, as evidenced by heightened levels of catechin and epicatechin detected in the brain of rats fed the grape seed extract enriched diet (Prasain *et al.*, 2009).

Table 2.1 summarizes the total phenolic (TPC), total flavanol (TFC), and total anthocyanin (TAC) contents of selected grape varieties estimated using spectrophotometric methods from previous studies.

Table 2. 1

Total phenolic, flavanol, and anthocyanin contents of grape seeds.

Variety	TPC (mg GAE/g)	TFC (mg/g)	TAC (mg CGE/100 g)	Reference
Ebizuru	8.8 ^F	1.3 QE ^F	ND	Poudel <i>et al.</i> , 2008
Ryukyuganebu	3.6 ^F	1.0 QE ^F	ND	Poudel <i>et al.</i> , 2008
Shiohtashibudou	13.6 ^F	5.5 QE ^F	ND	Poudel <i>et al.</i> , 2008
Shiragabudou	16.5 ^F	1.4 QE ^F	ND	Poudel <i>et al.</i> , 2008
Yamabudou	5.7 ^F	0.8 QE ^F	ND	Poudel <i>et al.</i> , 2008
Kadainou R-1	8.7 ^F	0.9 QE ^F	ND	Poudel <i>et al.</i> , 2008
Kadainou R-1 x Bailey Alicante A.	8.7 ^F	0.7 QE ^F	ND	Poudel <i>et al.</i> , 2008
Bailey Alicante A	17.9 ^F	0.9 QE ^F	ND	Poudel <i>et al.</i> , 2008
Muscat of Alexandria	54.9 ^F	1.0 QE ^F	ND	Poudel <i>et al.</i> , 2008
Merlot	105.7 ^D	122.7 CE ^D	NA	Bozan <i>et al.</i> , 2008
Cabernet	103.7 ^D	125.0 CE ^D	NA	Bozan <i>et al.</i> , 2008
Cinsault	88.1 ^D	97.1 CE ^D	NA	Bozan <i>et al.</i> , 2008
Papaz Karasi	154.6 ^D	179.4 CE ^D	NA	Bozan <i>et al.</i> , 2008
Ada Karasi	137.5 ^D	163.4 CE ^D	NA	Bozan <i>et al.</i> , 2008
Hamburg Muscat	104.4 ^D	105.7 CE ^D	NA	Bozan <i>et al.</i> , 2008
Alphonso Lavallee	105.3 ^D	123.3 CE ^D	NA	Bozan <i>et al.</i> , 2008
Okuzgozu	139.4 ^D	174.5 CE ^D	NA	Bozan <i>et al.</i> , 2008
Bogazkere	94.2 ^D	95.0 CE ^D	NA	Bozan <i>et al.</i> , 2008
Senso	79.2 ^D	89.2 CE ^D	NA	Bozan <i>et al.</i> , 2008
Kalecik Karasi	136.2 ^D	147.7 CE ^D	NA	Bozan <i>et al.</i> , 2008
Bronze Muscadine	19.2-32.6 ^{aF}	NA	1.2-8.7 ^{aF}	Pastrana-Bonilla <i>et al.</i> , 2003
Purple Muscadine	15.4-26.9 ^{bF}	NA	2.2-7.5 ^{bF}	Pastrana-Bonilla <i>et al.</i> , 2003
Cabernet Sauvignon	8.7 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Grenache Rouge	9.8 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Merlot	16.9 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Mandilaria	22.3 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Agiorgitiko	11.3 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Negoska	11.8 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Xinomavro	1.4 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Mavrodafni	4.0 ^F	NA	NA	Guendez <i>et al.</i> , 2005
Limnio	14.1 ^F	NA	NA	Guendez <i>et al.</i> , 2005

^a: range for five different varieties of bronze Muscadine grapes; ^b: range for five different purple Muscadine grapes.

TPC: total phenolic content; TFC: total flavanol content; TAC: total anthocyanin content; GAE: gallic acid equivalent; QE: quercetin equivalent; CE: catechin equivalent; CGE: cyanidin-3-glucoside equivalent; ^D: dry weight; ^F: fresh weight; NA: not analyzed; ND: not detected.

In general, TPC values were determined colorimetrically using Folin-Ciocalteu (FC) reagent. However, this method measures total reducing capacity, and thus may also measure other non-phenolic reducing agents, such as ascorbic acid. Of the grape varieties analyzed, Papaz Karasi seeds contained the highest total phenolic content of 154.6 mg gallic acid equivalents (GAE)/g on a per dry seed weight basis, followed by Okuzgozu at 139.4 mg GAE/g, and Ada Karasi at 137.5 mg GAE/g (Bozan *et al.*, 2008). Of the grape seeds reported on a per fresh weight basis, the seeds of Muscat of Alexandria contained the highest total phenolic content of 54.9 mg GAE/g seed (Poudel *et al.*, 2008), followed by 32.6 mg GAE/g in Summit Muscadine (bronze) and 26.9 mg GAE/g in Noble Muscadine (purple) grape seeds (Pastrana-Bonilla *et al.*, 2003). Under the same experimental conditions, the pulp, skin, and whole fruit of Summit Muscadine grape (**Table 2.2**) contained about 0.22, 5.41, and 3.10 mg GAE/g on a fresh weight basis, respectively, which is less than 1, 20, and 10% of that in the seeds (Pastrana-Bonilla *et al.*, 2003). Pastrana-Bonilla *et al.*, (2003) analyzed the TPC of ten different varieties of Muscadine grapes, five with bronze skins and five with purple skins. The seeds of the five bronze grapes had TPC value of 19.2-32.6 mg GAE/g, which was much higher than that of 3.0-5.5, 0.21-0.25, and 1.7-3.1 mg GAE/g determined in the skin, pulp, and whole fruit, respectively (**Table 2.2**). The total phenolics in the seeds were, on average, five times more concentrated than that in the skin and 80 times more concentrated than that in the pulp on a fresh weight basis (**Table 2.2**), suggesting that grape seeds may serve as an excellent source for dietary phenolic components. Notably, for the phenolics to be available to humans, the seeds must be ground prior to consumption in order to expose them to digestion; otherwise, whole seeds pass through largely undigested.

Table 2. 2Total phenolic content, anthocyanin content, and antioxidant capacity of whole fruit and parts of Muscadine grapes.^a

Variety	TPC (mg GAE/100 g)				TAC (mg CGE/100 g)				TEAC (μ M/g)			
	Seed	Skin	Pulp	Whole Fruit	Seed	Skin	Pulp	Whole Fruit	Seed	Skin	Pulp	Whole Fruit
Bronze Carlos	1920.3	545.6	25.1	307.9	1.2	2.6	ND	0.9	204.6	14.9	3.4	18.2
Early Fry	2367.2	303.0	21.3	169.1	8.7	2.5	ND	1.1	277.8	13.9	2.0	11.2
Fry	2356.3	332.2	23.8	199.0	4.6	0.8	ND	0.4	234.2	11.1	2.9	9.8
Summit	3258.7	541.0	22.3	309.7	3.1	2.8	ND	1.3	245.4	12.4	3.0	10.2
Late Fry	1986.0	348.9	24.0	252.3	3.7	2.0	ND	1.1	218.9	13.4	2.4	15.4
AV	2377.7	414.1	23.3	247.6	4.3	2.1	ND	1.0	236.2	13.1	2.7	13.0
Purple Paulk	1649.3	363.6	30.0	195.2	4.1	177.0	4.7	74.8	307.9	12.1	2.2	11.2
Cowart	2303.0	261.6	11.6	214.2	4.6	107.8	1.1	37.8	325.5	12.4	2.7	21.7
Supreme	1535.5	329.9	20.1	184.7	7.5	135.5	0.7	65.2	478.6	12.2	1.6	11.5
Ison	1726.2	365.0	26.0	218.9	4.6	174.5	1.9	69.5	284.9	13.3	2.1	15.9
Noble	2685.3	355.1	33.4	425.7	2.2	65.5	2.2	31.5	234.7	12.4	2.1	27.8
AV	1979.9	335.0	24.2	247.7	4.6	132.1	2.1	55.8	326.3	12.5	2.1	17.6

^a Data from Pastrana-Bonilla *et al.* 2003

TPC: total phenolic content; TAC: Total anthocyanin content; TEAC: trolox equivalent antioxidant capacity; GAE: gallic acid equivalent; CGE: cyanidin 3-glucoside equivalent; ND: not detected; AV: average value

The TPC of grape seed flours, oils, and extracts have been investigated. In 2006, Parry and others reported the TPC value of various fruit seed flours, including that of Pinot Noir and Chardonnay grape varieties. Chardonnay seed flour exhibited the highest TPC with 186.3 mg GAE/g flour, which was higher than that of Pinot Noir, and also higher than that of black and red raspberry, blueberry, and cranberry seed flours (Parry *et al.*, 2006). Bail *et al.*, analyzed the TPC values of nine European grape seed oils, and found that the highest TPC value was 0.1 mg GAE/g seed oil whereas the lowest was 0.06 mg GAE/g oil, suggesting that grape seed oils may not be a good source of phenolic compounds (2008). TPC of seed extracts of several Turkish grape varieties were evaluated by Yemis and others (2008) and Narince grape seed extract was shown to contain the highest total phenolic content with 587.3 mg GAE/g extract, while the lowest TPC value was 339.5 mg GAE/g extract. In 2009, using HPLC, Maier and others determined TPC of the intact grape seeds, seed oil press residues, and the seed flour which is the byproduct of grape seed-oil extraction of seven varieties of *Vitis vinifera*. The seeds had TPC values ranging from 188.7 to 1165.8 mg/kg dry matter (DM), which was statistically higher than the corresponding values for the seed flour, ranging from 147.4 to 492.7 mg/kg DM, suggesting that grape seed oil may contain significant levels of phenolic compounds, while the seed flour may also serve as a dietary source of phenolics (Maier *et al.*, 2009).

The highest reported total flavanol content (TFC) per fresh weight was found in Shiohtashibudou grape seeds, at a level of 5.5 mg quercetin equivalents (QE)/g (Poudel *et al.*, 2008), while Papaz Karasi contained the highest flavanol content on per dry seed weight basis, with 179.4 catechin equivalents (CE)/g (Bozan *et al.*, 2008). **Table 2.1** provides a summary of representative TFC values of grape seeds from several previous studies. The seeds, skins, and pulp of Pinot Noir, Pinot Meunier, and the Chardonnay grape were analyzed with HPLC (Mané

et al., 2007). The results showed that seed of the Pinot Noir, Pinot Meunier, and Chardonnay grapes had TFC values of 75.1, 101, and 57.6 mg/g, respectively, on a per fresh weight basis, which were much greater than that of 30, 24, and 21 mg/g detected in the corresponding skin fractions, or that of 0.45, 0.26, and 0.36 mg/g in the corresponding pulp samples (Mané *et al.*, 2007). It needs to be pointed out that these TFC values are estimated using catechin, epicatechin, epigallocatechin, or epicatechin-3-gallate as a standard reference compound (Mané *et al.*, 2007). In addition, Maier *et al.*, compared the TFC of seven grape seeds and their corresponding seed flours, the residue from seed oil press, and showed that the seeds contained 4.39-18.78 g TFC/kg and the seed flours had 2.5-13.5 g TFC/kg on per dry mass basis. The seed flour retained about 57-78.6% TFC after seed oil production, indicating that both whole seeds and seed flour, the by-product from seed oil preparation, may serve as a dietary source of flavanoids (2009).

Total anthocyanin content (TAC) has been quantified for grape seeds in a number of previous studies using a UV-visible spectrophotometer according to a pH-differential method. The highest reported total anthocyanin content (TAC) was found in Early Fry Muscadine (bronze) grape seeds (**Table 2.2**), with 8.7 mg cyanidin-3-glucoside equivalents (CGE)/100 g fresh seeds (Pastrana-Bonilla *et al.*, 2003). It was interesting that seeds of bronze Muscadine grapes had a TAC value ranging from 1.2 to 8.7 mg CGE/100 g fresh seeds, whereas that of purple grapes had a TAC range of 2.2-7.5 mg CGE/100 g fresh seeds (Pastrana-Bonilla *et al.*, 2003), suggesting that that TAC values of grape seeds could not be predicted by the pigmentation in grape skins, although anthocyanin is the pigment responsible for the purple color in both grapes and wine. The data, in **Table 2.2**, show that there were low levels of anthocyanins in the seed and no anthocyanins detected in the pulp of the bronze varieties of Muscadine grapes. The anthocyanin content was only slightly higher in the skins of these varieties on a fresh weight

basis (**Table 2.2**). In the purple-skinned varieties of Muscadine grapes, the seeds and pulp contained lower levels of anthocyanin at 2.2-7.5 and 0.7-4.7 mg CGE/100 g respectively, but the skins had high concentrations ranging from 65.5 to 177.0 mg CGE/100 g on a per fresh sample weight basis (Pastrana-Bonilla *et al.*, 2003). Total anthocyanin content in the seeds of purple Muscadines grapes was, on average, 1.3 times higher than that of the bronze grapes, while the skins of purple varieties had about 65 times more anthocyanins than that of bronze varieties (Pastrana-Bonilla *et al.*, 2003).

In summary, TPC values vary greatly in grape seeds and seed fractions, suggesting that genotype and environmental conditions during growth may alter the phenolic component and contents in grape seeds. For example, a recently published study by a group in China found that Asian varieties of grapes yielded, on average, seeds and skins with lower levels of various phenolic compounds than European varieties, and that hybrids of the two yielded grape seeds that fell within the range (Xu *et al.*, 2010). In addition, a study reported in 2010 found that organically grown grapes contained higher levels of anthocyanins, flavanols, and total phenolic content than conventionally grown grapes, suggesting that growing methods may also significantly affect the chemical composition of grapes, and therefore grape seeds (Mulero *et al.*, 2010). Overall, grape seeds appear to be an excellent source of phenolic compounds, and can contribute significantly to the economic value of grapes for the viticulture industry.

Phenolic Composition of Grape Seeds

In addition to TPC, the quantities of individual polyphenolic compounds have also been of interest to researchers because they may contribute to the overall and selected health beneficial effects differently. For example, they may have different antioxidant properties.

Catechins, anthocyanins, and other phenolic compounds such as gallic acid have been detected in grape seeds (**Fig. 2.1**) (Fuleki & da Silva, 1997; Kammerer *et al.*, 2004; Guendez *et al.*, 2005; Montealegre *et al.*, 2006; Maier *et al.*, 2009). **Table 2.3** shows the concentrations of major polyphenolic compounds reported in grape seeds; only a small part of the values from previous studies are included.

Table 2. 3

Polyphenolic composition of grape seeds.

Variety	CT	EC	ECG	EGCG	B1	B2	GA
Cabernet Sauvignon ^a	215 ^F	89.3 ^F	27.9 ^F	6.5 ^F	14.8 ^F	11.3 ^F	2.8 ^F
Grenache Rouge ^a	203 ^F	86.8 ^F	18.6 ^F	9.5 ^F	10.6 ^F	6.1 ^F	3.4 ^F
Merlot ^a	183 ^F	83.4 ^F	58 ^F	13.5 ^F	13.5 ^F	17.6 ^F	2.7 ^F
Mandilaria ^a	454 ^F	249 ^F	64.4 ^F	15.6 ^F	102 ^F	69.2 ^F	10.5 ^F
Agiorgitiko ^a	245 ^F	172 ^F	41.3 ^F	10.9 ^F	31.9 ^F	36.1 ^F	17.9 ^F
Xinomavro ^a	36.7 ^F	17.5 ^F	0.1 ^F	0.1 ^F	0 ^F	0.1 ^F	0.7 ^F
Limnio ^a	51.3 ^F	20.1 ^F	13.8 ^F	0.3 ^F	0 ^F	0.1 ^F	1.2 ^F
Turkish Varieties ^b	471-2580 ^D	249-1688 ^D	32-1150 ^D	79-255 ^D	56-194 ^D	41-160 ^D	NA
Spätburgunder ^c	376 ^D	612 ^D	92 ^D	NA	499 ^D	298 ^D	NA
Samtrot ^c	464 ^D	331 ^D	198 ^D	NA	207 ^D	273 ^D	NA
Müller-Thurgau ^c	217 ^D	206 ^D	80 ^D	NA	121 ^D	121 ^D	NA
Kerner	88 ^D	223 ^D	41 ^D	NA	61 ^D	116 ^D	NA
Schwarzriesling ^c	238 ^D	266 ^D	169 ^D	NA	91 ^D	122 ^D	NA
White grape ^d	12-50 ^D	11-31 ^D	1.3-6.7 ^D	NA	20-62 ^D	1.9-3.3 ^D	NA
Red grape ^d	8.2-27 ^D	6-21 ^D	3.2-7 ^D	NA	7.4-17 ^D	2.1-4.1 ^D	0.7-1.0 ^D
Vinifera ^e	25-244 ^D	24-193 ^D	NA	NA	11-62 ^D	29-93 ^D	NA
Hybrid ^e	21-155 ^A	23-284 ^A	NA	NA	3-60 ^A	9-106 ^A	NA
Labruska ^e	37-58 ^A	55-97 ^A	NA	NA	7-11 ^A	29-75 ^A	NA
Weisser Riesling ^f	79 ^D	67.5 ^D	45.8 ^D	NA	105.4 ^D	50.6 ^D	NA

References: ^a - Guendez *et al.* 2005; ^b - Bozan *et al.* 2008; ^c - Maier *et al.* 2009; ^d - Montealegre *et al.* 2006; ^e - Fuleki and Ricardo da Silva 1997; ^f - Kammerer *et al.* 2005.

All quantities expressed in mg/100 g seed. CT: (+)-catechin; EC: (-)-epicatechin; ECG: (-)-epicatechin gallate; EGCG: (-)-epigallocatechin gallate; B1: proanthocyanidin B1; B2: proanthocyanidin B2; GA: gallic acid; ^D: dry weight; ^F: fresh weight; ^A: air dried; NA: not analyzed.

Of the varieties reported on a dry weight basis, the highest concentration of catechin was found in the seeds of Okuzgozu variety at 2580 mg/100 g, and the highest epicatechin concentration was found to be 1688 mg/100 g dry mass in Senso seeds (Bozan *et al.*, 2008). Okuzgozu and Kalecik Karasi grape seeds had the highest epicatechin gallate (1150 mg/100 g) and epigallocatechin gallate (255 mg/100 g) contents, respectively (Bozan *et al.*, 2008), while the highest proanthocyanidin B1 and B2 content was observed in Spätburgunder grape seeds measuring up to 499 mg/100 g and 298 mg/100 g, respectively (Maier *et al.*, 2009). Of the varieties measured on a fresh weight basis, the most remarkable one was the seeds of Mandilaria variety that had the highest levels of catechin at 454 mg/100 g, epicatechin at 249 mg/100g, epicatechin gallate at 64.4 mg/100 g, epigallocatechin gallate at 15.6 mg/100 g, procyanidin B1 at 102 mg/100 g, and procyanidin B2 at 69.2 mg/100 g, along with the greatest total polyphenolic concentration and the second highest concentration of gallic acid at 10.5 mg/100 g (Guendez *et al.*, 2005). Catechin and epicatechin were, on average, the most abundant polyphenolic compounds in the seeds of the analyzed grape varieties. Of the rest of the compounds analyzed, proanthocyanidin B2 and epicatechin gallate were the next most abundant, depending on the variety of grape and method of estimation.

Grape parts also may differ in the concentrations of individual phenolic compounds (Monagas *et al.*, 2003; Kammerer *et al.*, 2004; Yilmaz & Toledo, 2004; Montealegre *et al.*, 2006; Iacopini *et al.*, 2008; Huang *et al.*, 2009). **Table 2.4** compares the levels of catechin, epicatechin, and procyanidin B1 in the seeds and skins of eleven grape varieties, including four red and six white.

Table 2.4

Polyphenolic composition of seeds and skins of red and white grape varieties.

Variety	Catechin		Epicatechin		Procyanidin B1	
	Seed	Skin	Seed	Skin	Seed	Skin
Weisser Riesling 2002 ^a	79.0 ^D	22.7 ^D	67.5 ^D	13.5 ^D	105.4 ^D	19.2 ^D
Riesling ^b	40.0 ^F	1.4 ^F	16.0 ^F	trace	62.0 ^F	1.2 ^F
Merlot ^b	24.0 ^F	2.5 ^F	21.0 ^F	1.3 ^F	17.0 ^F	2.1 ^F
Cabernet Sauvignon ^b	27.0 ^F	1.7 ^F	13.0 ^F	0.6 ^F	15.0 ^F	1.2 ^F
Chardonnay ^b	39.0 ^F	2.3 ^F	31.0 ^F	0.6 ^F	38.0 ^F	2.3 ^F
Sauvignon Blanc ^b	20.0 ^F	1.0 ^F	13.0 ^F	0.3 ^F	25.0 ^F	1.6 ^F
Moscatel ^b	35.0 ^F	1.6 ^F	12.0 ^F	0.3 ^F	33.0 ^F	2.1 ^F
Gewürztraminer ^b	50.0 ^F	1.9 ^F	15.0 ^F	0.8 ^F	46.0 ^F	4.8 ^F
Viogner ^b	12.0 ^F	trace	11.0 ^F	trace	20.0 ^F	trace
Cencibel ^b	8.2 ^F	2.2 ^F	6.0 ^F	0.8 ^F	7.4 ^F	2.2 ^F
Shiraz ^b	12.0 ^F	0.9 ^F	13.0 ^F	0.7 ^F	10.0 ^F	0.8 ^F

References: ^a - Montealegre *et al.* 2006; ^b - Kammerer *et al.* 2005.All quantities expressed in mg/100 g seed. ^D: dry weight; ^F: fresh weight.

The tested seeds exhibited higher levels of catechin, epicatechin, and procyanidin B1 than their corresponding skin samples on a per sample weight basis regardless of grape skin color (**Table 2.4**). This observation is supported by the results from a number of other studies (Monagas *et al.*, 2003; Yilmaz & Toledo, 2004). In 2003, Monagas and colleagues examined the levels of anthocyanins and flavanols in the skin, seeds, and wine of Tempranillo, Graciano, and Cabernet Sauvignon grapes. Phenolic compositions differed greatly between the skin and seeds of same grapes and between seeds from different grape samples (Monagas *et al.*, 2003). The seeds had higher levels of all detected flavanol and anthocyanin compounds than the corresponding skin samples. For instance, Tempranillo seeds contained (-)-epicatechin at a level of 0.62 mg/g, whereas the skin had a (-)-epicatechin concentration of 0.079 mg/g on a dry weight basis (Monagas *et al.*, 2003). Another study by Yilmaz and Toledo also detected higher levels of catechin in the seeds as opposed to the skins of Chardonnay and Merlot grapes (Yilmaz & Toledo, 2004). It was also reported from this study that seeds of both varieties had greater gallic acid concentrations (15 and 10 mg/100g dry seeds compared to 5 and 3 mg/100 g dry skin, respectively) for Chardonnay and Merlot grapes. It needs to be pointed out that a few studies reported the levels of quercetin, resveratrol, rutin, myricetin, cyanidin glucoside and other phenolic compounds in grape skins, but did not report their presence in the seeds (Pastrana-Bonilla *et al.*, 2003; Iacopini *et al.*, 2008; Huang *et al.*, 2009). This indicated that grape seeds may have unique phenolic composition compared to the skin parts and could be utilized for different beneficial effects.

Trans-Resveratrol

Trans-Resveratrol (*trans*-3,5,4'-trihydroxystilbene, sometimes referred to as resveratrol) is a polyphenolic compound naturally present in grape skins and seeds (**Fig. 2.1C**). *Trans*-resveratrol has been shown to have a number of health beneficial effects including inhibition of platelet aggregation, anti-inflammatory activity, antioxidant properties, capacity to reduce the risk of cancer and cardiovascular disease, and possible longevity promoting effect (Howitz *et al.*, 2003; Wood *et al.*, 2004; Yilmaz & Toledo, 2004; Li *et al.*, 2006; Iacopini *et al.*, 2008). The levels of *trans*-resveratrol in grape seeds and skin were investigated (Li *et al.*, 2006; Iacopini *et al.*, 2008). Extractable or available amounts of *trans*-resveratrol in the seeds and skin of 120 grape varieties grown in two years have been compared (Li *et al.*, 2006). Methanol at a solvent-solid ratio of 5 mL for each gram of frozen seeds was used for *trans*-resveratrol extraction at 25°C for 48 hours in dark. Results from this study, measured using HPLC-UV, showed that grape seeds contained high levels of *trans*-resveratrol (Li *et al.*, 2006). This study also showed that both variety type and growing conditions altered its level in seeds and skin. The level of *trans*-resveratrol varied from 1.06 to 17.03 µg/g in the tested grape seeds, and varied from 0.56 to 145.11 µg/g skin on per fresh weight (Li *et al.*, 2006). Importantly, seeds of some varieties of grape contained greater level of *trans*-resveratrol than their counterpart skin samples. Taken together, these data indicated that grape seeds and skin may serve as dietary sources for *trans*-resveratrol.

In contrast, a recent study detected no *trans*-resveratrol in the grape seeds, while significant level of *trans*-resveratrol was found in grape skin samples under the same experimental conditions (Iacopini *et al.*, 2008). Ethanol:water:hydrochloric acid (0.12 M) at 70:29:1 (v/v/v) was used for extracting resveratrol and the extraction was performed in 4 hours.

While the difference in grape varieties could be a possible explanation for the absence of extracted *trans*-resveratrol from grape seeds, it could also have been partially due to a different extraction method.

Antioxidant Properties

Antioxidative Properties of Grape Seeds

Grape seeds have been shown to have antioxidative properties (Jayaprakasha *et al.*, 2003; Janisch *et al.*, 2006; Kim *et al.*, 2006; Yilmaz & Toledo, 2006; Bozan *et al.*, 2008; Iacopini *et al.*, 2008; Poudel *et al.*, 2008). The seeds of *V. vinifera* variety Bangalore blue grapes grown in India were analyzed for their capacity to reduce Mo (VI) to Mo (V) and to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Jayaprakasha *et al.*, 2003). The seed extracts exhibited dose-dependent DPPH scavenging property and reducing power under the experimental conditions. In 2008, defatted seeds of eleven grape varieties grown in Turkey were extracted with acetone:water(70:30, v/v) containing 0.5% acetic acid at 50°C and the extracts were evaluated for their free radical scavenging activities (Bozan *et al.*, 2008). All eleven seed extracts showed significant ability to directly react with and quench peroxy (ORAC) and DPPH radicals (Bozan *et al.*, 2008). The greatest ORAC value was 3.0 mmol trolox equivalents (TE)/g dry seeds determined in the Okuzgozu grape seeds, and the lowest ORAC value was 1.4 mmol TE/g for Bogazkere seeds; a 2-fold difference (**Fig. 2.2**).

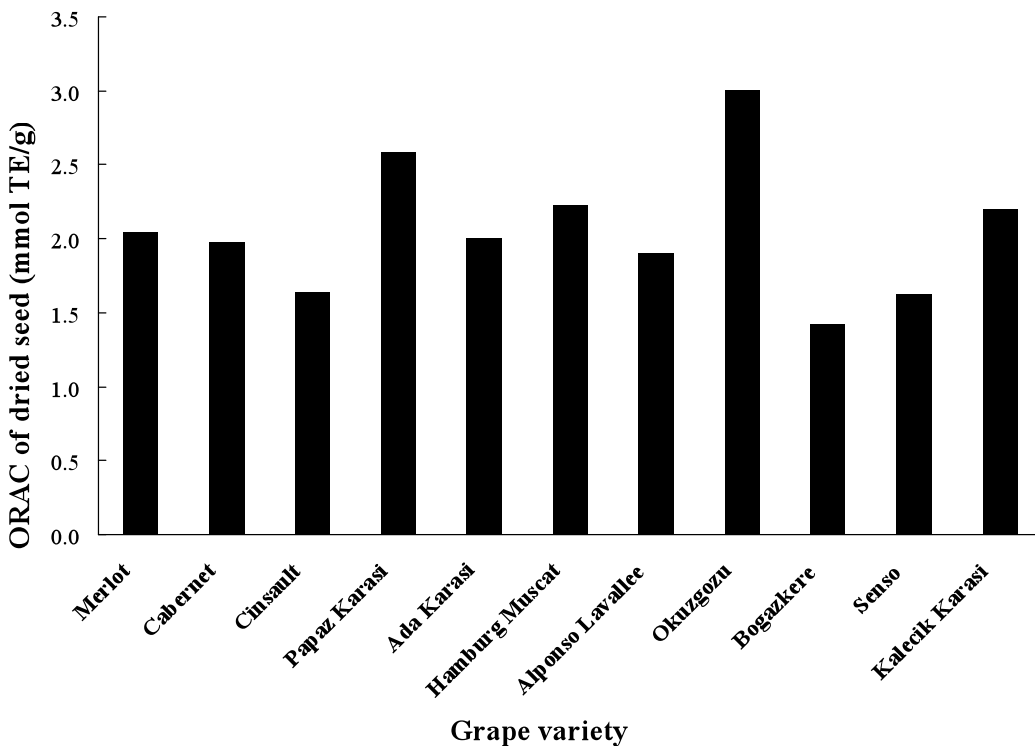


Figure 2.2

Oxygen radical absorbance capacity (ORAC) of grape seed extracts. Adapted from Bozan *et al.* (2008). Results were reported as millimoles of trolox equivalents per gram (mmol TE/g)

The DPPH radical scavenging capacity was reported using EC_{50} values, which is the required antioxidant concentration to quench 50% of the radicals in the system under the assay conditions. The Papaz Karasi grape seeds had the smallest EC_{50} value against DPPH radicals, which represented the greatest DPPH radical scavenging capacity, whereas the Okuzgozu seeds had an EC_{50} value of 2.89 $\mu\text{g/mL}$, the third strongest DPPH radical scavenging ability among the eleven grape seeds (Bozan *et al.*, 2008). DPPH radical scavenging capacity was also determined for five wild grapes in another study (Poudel *et al.*, 2008). Another study showed the DPPH \cdot and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS $^{\cdot+}$) scavenging capacities of 12 varieties of *V. vinifera* grape grown in Turkey (Yemis *et al.*, 2008). All tested grape seeds

showed significant scavenging capacity against both DPPH \cdot and ABTS $^{+\cdot}$. While the different grape varieties ranked differently in ABTS $^{+\cdot}$ scavenging and DPPH \cdot scavenging, both DPPH \cdot and ABTS $^{+\cdot}$ scavenging capacities were significantly correlated to the total phenolic contents of the seeds (Yemis *et al.*, 2008). In 2009, a study compared grape seed extract with ascorbic acid and chlorhexidine for their ability to directly react with and quench chemically generated ABTS cation radicals (Furiga *et al.*, 2009). The grape seed extract had the greatest ABTS $^{+\cdot}$ scavenging capacity under the experimental conditions. These results indicated that these grape seeds may differ in the content and compositions of their antioxidative components, which might have interacted with peroxy (ORAC), ABTS cation, and DPPH radicals in different manners under the assay conditions. It is also widely accepted that individual antioxidant activity assays differ in their determination principles and antioxidant activity estimation depends on the assays selected.

Grape seeds have also been compared with other grape parts for antioxidant properties. The pulp, peel, and seeds of red rose grape from a Chinese local market were compared for their ferric reducing/antioxidant power (FRAP) using FeSO $_4$ as the standard (Guo *et al.*, 2003). The seeds had a FRAP value of about 56 mmol/100 g, which was much greater than 0.49 and 11 mmol/100 g for pulp and skin, respectively, on a per fresh weight basis. In 2008, seeds and skins of ten native Tuscan and international red grape samples were compared for their DPPH radical scavenging abilities (Iacopini *et al.*, 2008). The IC $_{50}$ values, which are the required antioxidant concentration to quench 50% of the radicals in the assay mixtures under the experimental conditions, were estimated (**Fig. 2.3**).

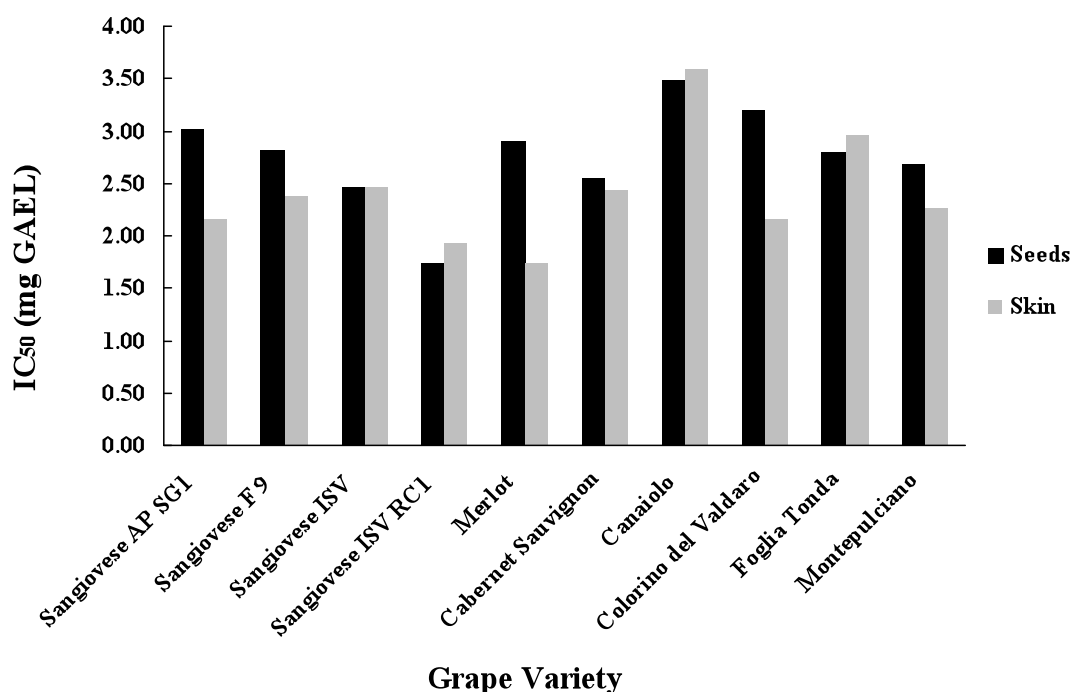


Figure 2.3

Antioxidant activity of grape seed and skin of different grape varieties native to Tuscan. Values were reported as IC₅₀ values in gallic acid equivalents per liter (GAEL), which was the concentration required to inhibit 50% of radicals (Iacopini *et al.*, 2008).

The antioxidants were extracted using ethanol:water:hydrochloric acid (0.12 M) (70:29:1, v/v/v). The seeds of Sangiovese clone ISV RC1 and the skin of Merlot grapes had the strongest DPPH radical scavenging capacity with the lowest same IC₅₀ value of 1.74 mg GAE/L. In 2006, Yilmaz and Toledo compared the peroxyl radical scavenging capacity (ORAC) for the seeds and skin of Merlot and Chardonnay grapes (Yilmaz & Toledo, 2006). The seeds had ORAC values of 345 and 638 µmol TE/g for Merlot and Chardonnay grapes, respectively, which were much higher than that of 70 and 103 µmol TE/g for the counterpart skin samples on a per dry weight basis (Yilmaz & Toledo, 2006).

Recently, Choi and Lee (2008) reported that a tocotrienol-rich fraction prepared from Campbell Early grape seeds had scavenging capacity against ABTS⁺ and DPPH[·], Fe²⁺ chelating activity, reducing power, and inhibition of linoleic acid oxidation. These results agreed with the observations from an earlier study showing that Chardonnay grape seed flour extract could suppress overall lipid peroxidation and prevent oxidative loss of longer chain ω -3 polyunsaturated fatty acids in fish oil (Luther *et al.*, 2007). The seed flour was the residue from seed oil preparation by cold-pressing. This study also demonstrated peroxyl radical scavenging capacity (ORAC) of Chardonnay seed flours, which was more than 6 times higher than the black raspberry seed flour on a per dry flour weight basis under the same assay conditions. DPPH[·] scavenging activity of the grape seed extract at a final concentration of 26 mg seed flour equivalent/mL was similar to that of the black raspberry seed extract, and greater than that observed for 50 ppm of the mixed tocopherol (Luther *et al.*, 2007). In addition, the cold-pressed Chardonnay and Pinot Noir grape seed flours were evaluated for their ORAC, DPPH[·] scavenging, and Fe²⁺ chelating capacities (Parry *et al.*, 2006).

Individual grape phenolic compounds have been investigated for their antioxidant properties in human low-density lipoprotein (LDL) (Janisch *et al.*, 2006). A reduced degree of LDL oxidation has been associated with a lower plaque formation in arteries (Stocker & Keaney, 2004). The required concentration for gallic acid, catechin, epicatechin and procyanidins B1, B2, B3, C1 and EB5 to achieve the maximum lag time measured as diene formation was determined in the study. Procyanidin EB5 was the most effective compound to prevent lipid peroxidation in the LDL under the experimental conditions (**Fig. 2.4**).

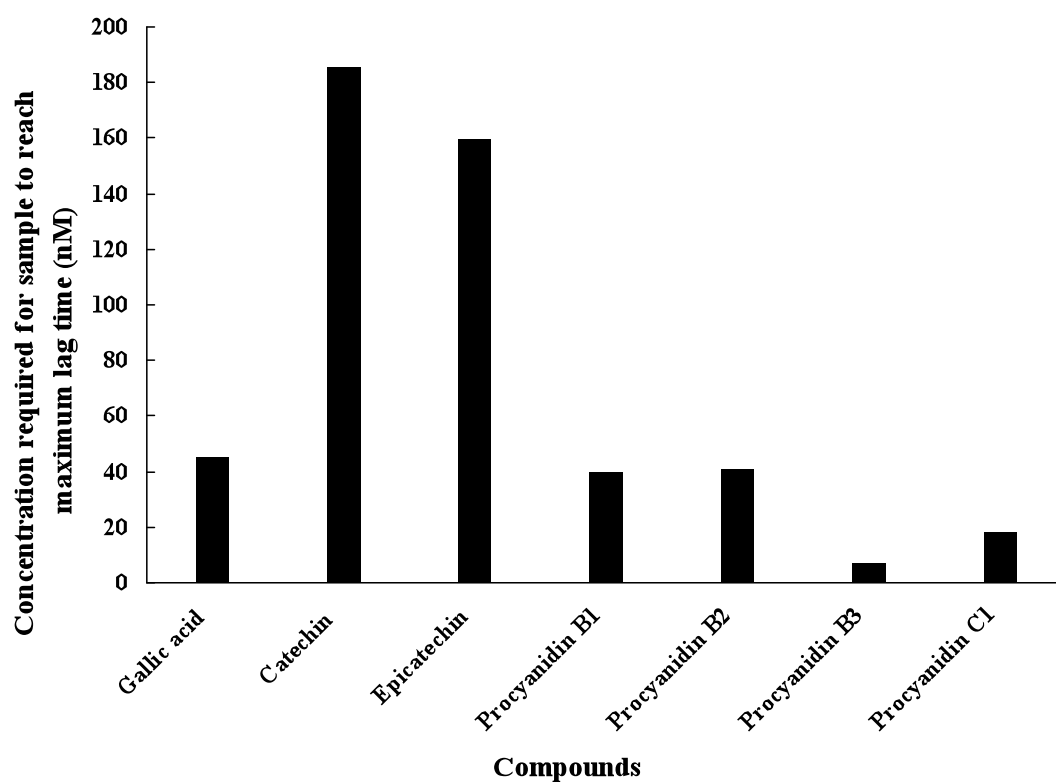


Figure 2. 4

Inhibitory effects of individual grape phenolic compounds on lipid peroxidation in human LDL. Results were expressed in nanomolar of each phenolic compound required to reach maximum lag time (adapted from Janisch *et al.*, 2006). The lag time was determined by photometrically tracking accumulation of conjugated diene formation through absorbance at $\lambda = 234$ nm.

The ability of these compounds to quench hydroxyl and peroxy anion radicals generally followed the order of suppressing LDL oxidation (Janisch *et al.*, 2006). Earlier in 1991, catechin, epicatechin, epicatechin gallate, procyanidins B2 and B5 and C1, procyanidin B2 gallate, as well as procyanidin trimer 2 and trimer 3 showed peroxide anion radical scavenging capacity at pH 7.5 and pH 9.0 conditions (da Silva *et al.*, 1991). In 2008, individual grape phenolic compounds such as catechin, epicatechin, rutin, *trans*-resveratrol, and quercetin were shown to have scavenging capacity against DPPH· and peroxy nitrite (Iacopini *et al.*, 2008). In addition, gallic acid and catechin were reported for their scavenging capacity against DPPH·, peroxide anion ($O_2^{\cdot-}$) and hydroxyl (HO^{\cdot}) radicals, and their reducing power (Spranger *et al.*, 2008).

Finally, *trans*-resveratrol was compared with other grape phenolic compounds for antioxidant properties. *Trans*-resveratrol exhibited a peroxy radical scavenging capacity (ORAC value) of 29.06 μmol trolox equivalent (TE)/mg, while catechin had an ORAC value of 20.53 μmol /mg under the same assay conditions, which was followed by epicatechin, gallic acid, and ellagic acid with a range of ORAC value from 20.53 to 3.88 μmol TE/mg (Yilmaz & Toledo, 2004). However, *trans*-resveratrol had a weaker DPPH radical scavenging capacity than quercetin, catechin, epicatechin, and rutin according to their IC_{50} values against DPPH radicals (Iacopini *et al.*, 2008).

It needs to be pointed out that grape phenolic antioxidants have shown other beneficial effects besides their antioxidative properties. These beneficial effects may include but are not limited to anti-proliferative activity against cancer cells (Parry *et al.*, 2006; Choi & Lee, 2008), antibacterial activity (Jayaprakasha *et al.*, 2003; Luther *et al.*, 2007), lifespan extension (Howitz *et al.*, 2003; Wood *et al.*, 2004), and prevention of

cataract formation (Yamakoshi *et al.*, 2002). These beneficial properties may not be mediated by their antioxidant activities, but rather by other metabolic pathways.

Effects of Post-Harvest Treatments on Grape Seed Antioxidants

Effects of Thermal Treatment

The bioavailability of bioactive food factors is critical for their beneficial effects. The bioavailability depends on their original concentration in the raw ingredients and changes during post-harvest treatments, such as chemical and biochemical reactions during storage and ingredient processing, as well as their interactions with other components during food formulation and processing. Effects of thermal treatment on antioxidant availability in grape seeds has been investigated (Kim *et al.*, 2006). Whole and powdered grape seeds (*V. vinifera*, Campbell Early) were heated at 50, 100, 150, or 200 °C for 10, 20, 30, 40, 60, 90, and 120 minutes. These seed preparations were compared to the control, which are the seeds without thermal treatment, for their TPC and antioxidant properties. As shown in **Table 2.5**, TPC values decreased in both whole and powdered grape seeds in a temperature and time dependent matter except the powdered seeds kept at 50 °C, indicating the possible loss of phenolic components due to thermally induced chemical reactions, such as oxidation.

Table 2. 5Effects of thermal treatment on total phenolic content (mM TAE) of whole and powdered grape (*V. vinifera*, Campbell early) seeds.^a

Temperature (°C)	Heating Time (min)							
	0	10	20	30	40	60	90	120
WGSE								
50	0.380	0.317	0.260	0.303	0.300	0.313	0.442	0.330
100	0.380	0.326	0.347	0.414	0.407	0.520	0.458	0.390
150	0.380	0.392	0.348	0.444	0.575	0.484	0.358	0.319
200	0.380	0.254	0.189	0.163	0.115	0.179	0.179	0.113
PGSE								
50	0.380	0.344	0.332	0.451	0.424	0.444	0.400	0.515
100	0.380	0.555	0.296	0.359	0.375	0.378	0.418	0.185
150	0.380	0.340	0.417	0.427	0.483	0.407	0.319	0.196
200	0.380	0.190	0.185	0.269	0.160	0.151	0.067	0.064

^a Referenced from Kim *et al.* 2006

TAE: tannic acid equivalents; WGSE: whole grape seed extract; PGSE: powdered grape seed extract.

It should also be noted that powdered grape seeds demonstrated a higher TPC value than the whole seed counterparts at all time points when kept at 50 and at 100 °C for 10 min, but the whole seeds had a greater TPC value than the powdered seed counterparts when they were kept at 150 and 200 °C for 10-120 min, or at 100 °C for 20-120 min. These results suggest that mild thermal treatment, such as heating at 50 °C for a short time period, may enhance the extractable or available level of phenolics in the powdered grape seeds. This also suggests that the seed matrix may protect phenolic compounds during thermal treatment. This observation may be explained by the overall effects of thermal cleavage of associations between phenolics and the seed matrix, and the increased surface area of the powdered seeds (Meyer *et al.*, 1997; Cheng *et al.*, 2006; Kim *et al.*, 2006). Heat treatment has been shown to possibly increase release of phenolic compounds as it may convert insoluble bound phenolic compounds into soluble phenolic compounds (Kim *et al.*, 2006; Moore *et al.*, 2009).

Thermal treatment also altered the antioxidant property of grape seeds (Kim *et al.*, 2006). As shown in **Fig. 2.5**, increase of the extractable DPPH radical scavenging capacity was observed in whole and powdered grape seeds kept at 50 and 100 °C for 60 and 120 min, while heating at 200 °C for 60 and 120 min decreased DPPH radical scavenging activity in whole and ground seeds (Kim *et al.*, 2006). Kim and colleagues (2006) also reported the alteration of reducing power of grape seeds by thermal treatment.

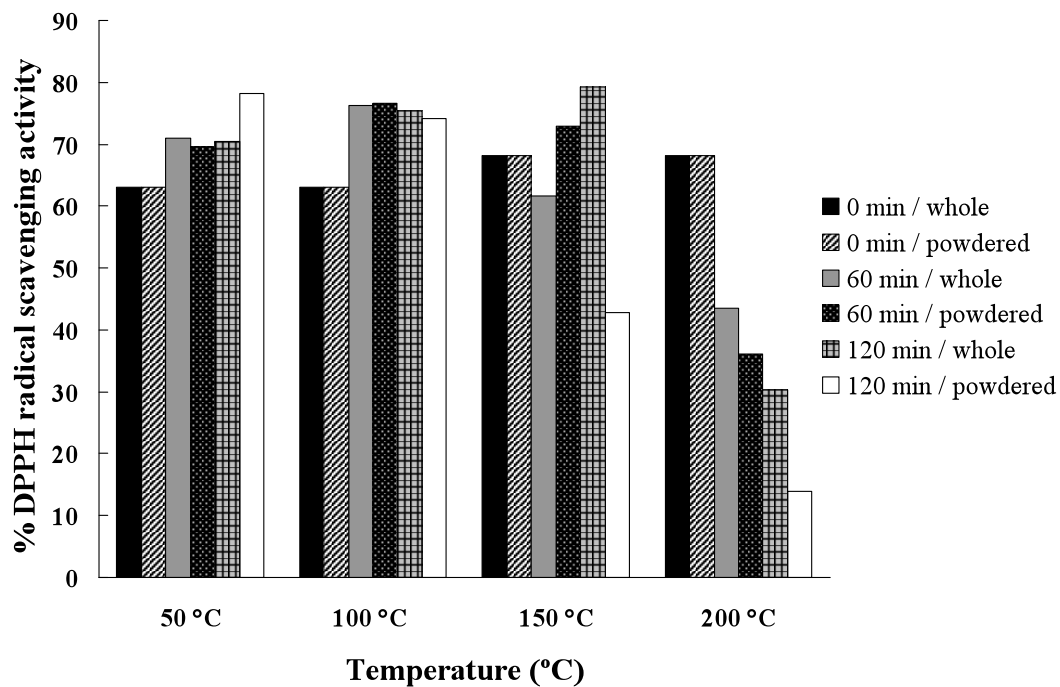


Figure 2. 5

Effects of thermal treatment and particle size on antioxidant stability in whole and powdered grape seeds (adapted from Kim *et al.*, 2006). The radical scavenging activity was estimated by the formula: % DPPH radical scavenging activity = $(1 - \text{sample absorbance} / \text{control absorbance}) \times 100\%$.

Table 2. 6Effects of different extraction times and crushing on total phenolic, anthocyanin, and flavanol contents of grape seed extracts.^a

Extraction Time	Total Phenolics (mg GAE/L)	Anthocyanins (mg ME/L)	Flavanols (mg CCE/L)
Cab. Sauvignon (whole seeds)			
1 min	565	718.0	0.0
1 h	686	696.0	0.0
4 h	771	793.8	0.0
24 h	737	705.8	8.9
165 h (\approx 7 days)	890	746.4	0.0
Cab. Sauvignon (crushed seeds)			
1 min	1780	791.7	133.7
1 h	1868	879.9	122.7
4 h	1930	867.8	140.9
24 h	2015	856.2	156.0
165 h (\approx 7 days)	2138	775.2	167.4
P. Sirah late (whole seeds)			
1 min	1115	1708.2	5.9
1 h	1136	1483.6	0.0
4 h	1163	1558.9	5.5
24 h	1183	1477.3	21.5
165 h (\approx 7 days)	1367	1564.7	99.4
P. Sirah late (crushed seeds)			
1 min	1741	1337.2	93.4
1 h	1820	1644.2	101.8
4 h	1966	1463.4	137.6
24 h	1964	1513.2	173.6
165 h (\approx 7 days)	2094	1344.0	168.0

^a Data from Meyer *et al.* 1997

GAE: gallic acid equivalents; ME: malvin equivalents; CCE: catechin equivalents.

Effects of Particle Size

Particle size of the botanical materials including food and nutraceutical ingredients may affect the stability of their important components during storage and post-harvest treatments such as ingredient and food processing procedures (Cheng *et al.*, 2006). In 2006, Kim and others theorized that reduction of particle size, through the grinding of grape seeds, might contribute to the increased available amount of total phenolics kept at 50 °C (see *Effect of Thermal Treatment* section). In addition to this observation, Meyer and colleagues reported that crushed seeds might have higher levels of extractable total phenolics, benzoic acids, flavanols, and cinnamates, but not anthocyanins, than their whole seeds counterparts (1997). Under the same analytical conditions, crushed Cabernet Sauvignon grape seeds had a TPC value of 1780 mg GAE/L and the TPC was 565 mg GAE/L for the whole seeds (**Table 2.6**).

Effects of Storage Conditions

Storage conditions have been found to affect antioxidant properties in grapes and grape seeds (Hatzidimitriou *et al.*, 2007; Romero *et al.*, 2008). Hatzidimitriou and colleagues analyzed the effects of storage at three different relative humidity (RH) levels, 33, 53, and 75%, on TPC of grape seed extracts (2007). TPC decreased from 438 to 327 and 438 to 344 mg GAE/g dry extraction respectively for the grape seeds kept at 33 and 53% RH in 50 days, and dropped from 438 to 234 mg GAE/g for seeds stored at 75% RH (Hatzidimitriou *et al.*, 2007). DPPH[•] scavenging abilities of the grape seed extracts were slightly reduced at all tested RH conditions. Interestingly, storage at either higher RH level

or for longer time could enhance the gallic acid level in the grape seeds, but would reduce both catechin and epicatechin contents (Hatzidimitriou *et al.*, 2007).

Considerations in Antioxidant Property Estimation for Grape Seeds

Many factors may alter the overall estimation of antioxidant properties of grape seeds. It is widely accepted that mistakes made during sample preparation cannot be corrected in the later analytical steps. In 2005, Pinelo and others investigated the effects of solvent, temperature, and solvent-solid ratio on estimation of total phenolic content and radical scavenging capacity of grape pomace, stem, seeds, and skin. The extraction temperature and solvent-solid ratio were critical in the extraction efficiency of phenolic antioxidants (Pinelo *et al.*, 2005). It was also noted that methanol was most effective for phenolic extraction, while ethanol extracted the highest level of soluble material under the experimental conditions, suggesting the importance of solvent type in antioxidant property estimation. This conclusion was supported by findings from a recent study (Yilmaz & Toledo, 2006). Results from this study showed that methanol, ethanol, and acetone with different levels of water differed in their capacities in extracting phenolic components from grape seeds and skin (Yilmaz & Toledo, 2006). The critical role of temperature and solvent-solid ratio on phenolic extraction from grape seeds was confirmed by a kinetic study performed by Bucić-Kojić and colleagues (2007). In addition, pH and particle size might alter the extraction efficiency of phenolic antioxidants from grape seeds (Janisch *et al.*, 2006; Makris *et al.*, 2007; Bucić-Kojić *et al.*, 2007).

Summary

Grape seeds and seed fractions may serve as dietary source of natural phenolic antioxidants, such as flavanols, anthocyanins, and simple phenolic acids. Genotype, growing conditions, and post-harvest treatments may alter the content of antioxidants in grape seeds. This may be a challenge in developing grape seeds-based nutraceutical ingredients for human utilization. Multi-mechanisms such as radical scavenging and chelating reactions may be involved in the antioxidative actions of these phenolic compounds. Additional research is required to investigate their health beneficial effects and possible side effects to promote their application in improving human health.

Incorporation Into Functional Foods

Functional Foods: An Overview

The study of functional food is a popular and constantly evolving area of food science. The term functional food has been defined by many different organizations worldwide. There is no universally accepted definition of this term, but the International Food Information Council (IFIC) and the Institute of Food Technologists (IFT) define functional foods as a food or food component that has health beneficial qualities beyond basic nutrition (International Food Information Council, 2009). More specifically, an IFT report extended the definition by stating that functional foods “provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects” (Institute of Food Technologists, 2009). The American Dietetic Association (ADA), on the other hand, believes all foods are functional to a certain extent.

In this definition, functional foods are those “that include whole foods and fortified, enriched, or enhanced foods have a potentially beneficial effect on health when consumed as a part of a varied diet on a regular basis at effective levels” (American Dietetic Association, 2009).

Political bodies, however, have been slow to resolve the controversy of defining functional foods. The United States Food and Drug Administration (FDA) offers no definition regarding functional foods. There is also no regulatory framework for foods that are marketed as functional food in the United States, either. In fact, Japan is the only country in the world that formally regulates functional foods as a distinct category (American Dietetic Association, 2009). The phrase “functional food” was first used in the 1980s in Japan to refer to food fortified with special constituents that had positive physiological effects (Kwak & Jukes, 2001). As functional foods have become more and more popular in recent years, there has been increasing pressure on the FDA to define functional food more clearly and regulate this \$20.5 billion market (Burdock *et al.*, 2006). A similar situation exists in the European Union (EU). Under EU Food Law, no framework for functional food regulation is available. Thus, the recent years have seen calls for more definition and regulation of functional food in Europe, as well (Coppens *et al.*, 2005).

Antioxidants in Functional Foods

Many nutrients have been added to functional foods with the purpose of improving their health beneficial properties. Antioxidants, specifically, have been successfully incorporated in a multitude of functional food products. Tortillas, for example, have been

developed into a functional food, through the addition of antioxidant-containing bean flours. In an experiment conducted by Anton and colleagues, flours were prepared from four varieties of bean (red, navy, pinto, and black) that were subsequently made into tortillas. In comparison to a wheat flour control, the bean flour tortillas were found to have higher levels of protein, total phenolic content (TPC), DPPH radical scavenging capacity, and ABTS cation radical scavenging capacity (Anton *et al.*, 2008).

Nutrients added to functional foods, however, are not always successful in increasing the overall intended health-beneficial properties of the product. An example of this can be found in a study of two foods, pan bread and sugar-snap cookies, which were supplemented with the food additives red palm olein (RPOL) and red palm shortening (RPS), with the intention of increasing the vitamin E content of these food products (Al-Saquer *et al.*, 2004). The results of this experiment showed that the total tocopherol content and the total tocotrienol content of the breads (whole-wheat, brown, and white) were substantially lowered in those containing 75% RPOL (192 and 407 mg/kg fat, respectively) or RPS (101.4 and 300 mg/kg fat, respectively), when compared to the bread samples made solely with control shortening that had a TPC of 325.0 mg/kg fat and a total tocotrienol content of 468 mg/kg fat. Similarly, total vitamin E content of the cookies containing 100% RPS (367.3 mg/kg fat) and 100% RPOL (489.9 mg/kg fat) was significantly lower than that of the bread containing 100% control shortening (781.6 mg/kg fat) (Al-Saquer *et al.*, 2004).

A number of factors influence the effectiveness of antioxidant properties in functional foods containing added antioxidants. A study, for example, conducted by Moore and colleagues (2009), analyzed the effect of specific processing conditions on the antioxidant properties of a whole-wheat pizza crust functional food model. The research looked into the

effects of these conditions on ORAC, hydroxyl radical scavenging capacity (HOSC), DPPH radical scavenging capacity, ABTS radical scavenging, TPC, and ferulic acid content, of whole-wheat pizza crust. The factors that were studied included bran particle size, dough fermentation time, and baking time/temperature. Results showed that bran particle size had no substantial effect on antioxidant properties. Similarly, little difference was noted when dough fermentation time increased (except HOSC which increased a maximum of 28%). It was noted that as baking temperature increased, from 204 °C to 288 °C with a 7 min bake time, all antioxidant properties increased up to 82%. An increase in bake time from 7 to 14 min at 204 °C increased some of the antioxidant properties. These results show that the functional food model (whole-wheat pizza crust) may have an increased antioxidant availability as dough fermentation time, baking time, and baking temperature are increased (Moore *et al.*, 2009).

Bread as a Functional Food Model

Bread has been shown to be an effective model for a variety of functional foods including: antioxidants, fatty acids, anti-phytases, gluten, γ -aminobutyric acid (GABA), and fiber. Ingredients of bread can be substituted with little negative effect on rheological properties or sensory quality (Saiz *et al.*, 2007). Flour has been substituted with chickpea, amaranth, quinoa, buckwheat, grape seed, flaxseed, barley, rice bran, wheat bran, sugarcane bagasse, carob fiber, inulin, and pea fiber (Gill *et al.*, 2002; Sangnark & Noomhorm, 2004; Penella *et al.*, 2008; Conforti & Cachaper, 2009; Hu *et al.*, 2009; Lin *et al.*, 2009; Coda *et al.*, 2010; Peng *et al.*, 2010). The novel bread products all were considered acceptable by sensory panels, indicating the possibility of commercial applications of bread as a functional food.

Additives in Bread Models

Antioxidants

Increased levels of antioxidants in bread were achieved with addition of different ingredients as well as novel processing methods. Addition of a chickpea, amaranth, quinoa, and buckwheat flour blend to a bread product, resulted in increased level of phenolics (Coda *et al.*, 2010). The non-conventional bread product also underwent sourdough fermentation and exhibited the highest levels of phenolics, followed by non-conventional bread baked under normal conditions, wheat bread exhibited the lowest levels. Non-conventional bread baked under both conditions also exhibited high DPPH-radical scavenging ability. Sourdough fermentation produces γ -aminobutyric acid (GABA), which functions as an anti-hypertensive, diuretic, tranquilizer, and prevents diabetes (Coda *et al.*, 2010).

Wheat flour was substituted with unhusked and husked buckwheat. Husked buckwheat bread contained more insoluble β -glucan, which provides an immunostimulating effect. Sensory analysis, using the 7 point hedonic scale where 1, 4, and 7 respectively representing strongly dislike, neither like nor dislike, and strongly like, showed that all three breads (husked, unhusked buckwheat, and wheat breads) were acceptable to consumers, and both husked and unhusked buckwheat breads scored higher in flavor and mouth-feel than the wheat bread. The flavanoids rutin and quercetin were found in substantial quantities in husked buckwheat bread, and in smaller quantities in unhusked buckwheat and wheat bread products. The buckwheat-enhanced bread also showed enhanced antioxidant properties, especially unhusked buckwheat bread product (Lin *et al.*, 2009).

Addition of grape seed extract to white bread resulted in increased antioxidant activity and decreased levels of the detrimental glycation end-product in bread, *Nε*-(carboxymethyl) lysine (CML). CML is considered a toxin in food and a biomarker associated with oxidative stress, atherosclerosis and diabetes. The grape seed bread showed little change in the sensory analysis. The baking process reduced antioxidant activity in the bread by 30 to 40 % (Peng *et al.*, 2010).

Fatty Acids

Quinoa and flaxseed (linseed) bread were prepared and compared for physiochemical properties and underwent a sensory analysis. The flaxseed bread was higher in both lipid level and caloric value. Both breads were low in trans-fatty acids, while flaxseed bread exhibited higher levels of saturated, monounsaturated, polyunsaturated, omega-6 and omega-3 fatty acids, as well as a lower omega-6 to omega-3 ratio. However, quinoa bread was found to be low in saturated fatty acids. Additionally, consumers preferred quinoa bread to flaxseed bread. During the baking process, fatty acids in both flaxseed and quinoa were lost from the original ingredients (Calderelli *et al.*, 2010).

Fiber

While the classification of fiber as a functional food is contested, a significant amount of research has been done on the subject, especially with bread as the model. Substitution of high-fiber grains can increase fiber content in bread. Barley contains high levels of soluble (digestible) fiber, with higher fiber content in waxy barley flour as

opposed to regular barley flour. However, substitution of wheat with barley flour decreased loaf volume and altered the color, firmness, and texture of the loaves. The addition of barley yielded poor quality bread products. Changes were dependent on barley variety (Gill *et al.*, 2002). Wheat bread has also been supplemented with carob fiber, inulin and pea fiber as sources of fiber. Carob and pea fiber led to softness in the bread crumb, and the breads supplemented with these two fibers were judged as acceptable. All three sources of fiber could be used to increase dietary intake. The level of soluble dietary fiber was greatly increased with inulin-added bread. The fiber with the greatest potential for increasing fiber intake, balanced with consumer acceptability was carob fiber (Wang *et al.*, 2002).

Additionally, different processing methods can lead to more nutritious functional food products. Extrusion cooking allows for improvements in nutritional quality of dietary fiber. Extrusion is most commonly used to produce breakfast cereals, and involves heating food under a high degree of pressure, then pushing foodstuffs through a series of pores. The use of extruded flour produced more favorable bread than using cooked flour (Gill *et al.*, 2002). Izydorczyk and others found that decreased particle size increased the percentage of water soluble beta-glucans and arabinoxylans, increased starch damage, and increased the level of free phenolics in fiber-enriched breads (2008).

Addition of fiber to bread products may have negative effects on the nutritive and sensory properties; however, other compounds can be added to minimize these negative effects. Phytic acid is a compound commonly found in plant seeds and grains that forms insoluble complexes with cations, decreasing the bioavailability of nutrients like calcium, magnesium, and potassium. The combination of adding bran and phytate-degrading

enzymes allows for the increased fiber from bran without the negative effects of the increased amounts of phytate from the bran (Penella *et al.*, 2008). Sangnark and Noomhorm (2004) found that combining sucrose ester and fiber allowed addition of fiber without the great losses in consumer acceptability. Sugarcane bagasse was used for addition of dietary fiber, and sucrose ester was added as an emulsifier to increase the proportion of dietary fiber in white pan bread. Addition of fiber decreased loaf volume, increased firmness, and negatively affected crumb color and bread texture, while addition of sucrose positively influenced bread properties.

Diet and Health Effects

Supplementation

With rising health care costs and research indicating that diet is directly correlated with the incidence of disease, Americans have focused more attention on consuming their daily nutrients through functional foods (Milner, 2000). However, supplementation along with functional foods can provide a means for Americans to get the health benefits of these nutrients and antioxidants within their diet.

The major determinants of the aging process are environmental and lifestyle factors, particularly diet. With aging, the likelihood of disease is greater, and high levels of oxidative stress and free radicals can cause many chronic illnesses, including arthritis, cardiovascular disease, and cancer (Meydani, 2000). Recent research indicates that certain nutritional supplements can counteract or minimize the effects of these diseases. Many vitamins, including vitamin E, have been found to have antioxidative properties. Research has shown that vitamin E is often supplemented with functional foods, and when

consumed it can have positive cardiovascular and immune effects in the elderly. In one study conducted, healthy subjects over the age of 60 who consumed 800 IU of vitamin E over a one-month period were found to have reduced production levels of prostaglandin ES (PGE2), a lipid based mediator molecule, and plasma lipid peroxide concentrations (Meydani, 2000). The vitamin E supplementation was also shown to enhance cell-mediated immunity.

The preventive effects of vitamin supplements on age-related diseases were also observed in a study conducted by Zandi and colleagues (2004). In the study, researchers examined the relationship between vitamins C, E, and B-complex supplements and Alzheimer's disease. The study was designed as a cross-sectional study of 4740 elderly respondents, with notes made of their dietary supplemental use over a 5-year period. The results showed that vitamin E used in combination with vitamin C exhibited the greatest protective effects against Alzheimer's disease. These results support previous study findings that show that vitamin supplementation plays a key role in reducing the occurrence of age-related diseases as a result of its antioxidative properties.

Antioxidants can also be supplemented into diets, and reduce oxidative stress in the body. For example, in one study, patients with Type-II diabetes were given a 200-mg α -tocopherol supplement over a two-month period. After the two-month period, the antioxidant supplementation was found to have decreased blood-lipid peroxide concentrations without affecting antioxidative activities of the patients (Park & Choi, 2002).

In other studies, antioxidants were supplemented into whole foods and their health benefits were studied (Vattem *et al.*, 2005). Fruits and vegetables have been found to

decrease the incidence of disease, which has increased the desire to supplement diets with the phenolic compounds that these foods contain. There is also evidence that suggests that phenolic phytochemicals in whole foods are more beneficial to human health than individual phenolic phytochemicals alone because of differences in bioavailability. As a result, more research has been conducted on how phenolic compounds can be added into a wider range of foods. For example, cranberries are a natural source of phenolic compounds with antioxidants that have been shown to have positive effects against urinary and cardiovascular diseases (Yan *et al.*, 2002). By contributing their phenolic compounds, cranberries might be used to enrich other functional foods and promote health benefits (Vattem *et al.*, 2005).

Another instance in which antioxidants were found to supplement diets can be seen in tea extracts. Researchers sought to determine whether green tea polyphenols could exert LDL-resistant effects in the body. LDL can react with free radicals and become oxidized. In its oxidized state, LDL can damage tissue and underlying smooth-muscle cells, and inflame arteries, which can result in atherosclerosis (Russell, 1999).

In a study by Miura and others (2000), male subjects were all asked to follow a prescribed dietary regimen for two weeks. After the two week period, they were divided into a control and experimental group, with the experimental group consuming 300 mg of green tea polyphenol extract twice a day for a week. After the trial, it was found that supplementation with green tea polyphenols significantly increased LDL resistance to in vivo oxidation, and prevented antioxidative vitamins from being depleted. The polyphenols were found to be useful in decreasing the risk of cardiovascular disease (Miura *et al.*, 2000).

In a study conducted in Finland, researchers determined the relationship between flavanoid intake and the incidence of lung cancer (Knekt *et al.*, 1997). The cross-sectional study involved 9959 men and women from the ages of 15 to 99. After 24 years, researchers found that the mean flavanoid intake was 4.0 mg per day with intake amounts ranging from 0 to 41.4 mg. Those subjects who consumed the highest level of flavanoids in their diet, which was greater than 4.8 mg/day of flavanoid for men and greater than 5.5 mg/day flavanoid for women, reduced their risk for lung cancer by 50%. Specifically, those subjects who consumed apples as part of their diet had a decreased risk for lung cancer. Overall, it was found that there was an inverse relationship between flavanoid consumption and the risk for lung cancer (Knekt *et al.*, 1997).

Other studies have investigated the health benefits of diets enriched with antioxidants. In a study conducted by Rebrin and others (2005), the effects of two different dietary mixtures, one consisting of vitamin C, vitamin E, L-carnitine, and lipic acid and another consisting of vitamin C, vitamin E, and micronutrients with bioflavanoids, polyphenols, and carotenoids, were studied. The first dietary mixture was fed to a group of mice for 8 months, and the second dietary mixture was fed to another group of mice for 10 months. Both diets were found to have reductive effects in plasma (Rebrin *et al.*, 2005). The first dietary mixture was found to have little effect on the glutathione (GSH) redox status in tissue homogenates or mitochondria. However, for the second dietary mixture, gender and tissue specific effects on the glutathione redox were observed. For example, there was an increase in serum cysteine concentrations in female mice, while GSH elevation occurred only in the homogenates of male kidney and skeletal muscles.

Functional Foods

Like dietary supplements, functional foods have been shown to have health benefits. The components of functional foods may help to reduce the risk of disease by cooperative and synergistic action (Shahidi, 2004). However, some antioxidants, such as β -carotene, have been found to have no effect or even be detrimental in supplement form because of a complex synergistic nature (Omenn *et al.*, 1996). Consequently, functional foods may provide a physiologically advantageous combination of antioxidants. Several types of functional foods have been developed to fulfill a variety of needs. These foods are also varied in the matrix used to acceptably deliver the as target nutrients.

By incorporating health beneficial compounds, commonly consumed food may be modified into healthier products. Some of these functional foods have been found to have significant physiological effects. In one study, biscuits were enriched with vitamin B12, folic acid, vitamin C, and prebiotic fiber (Boobier *et al.*, 2007). Subjects consumed four biscuits per day in conjunction with their normal diet. After 28 days of eating the biscuits, the levels of homocysteine and glucose in blood plasma, two factors that have been linked to cardiovascular diseases, decreased.

Several studies have incorporated antioxidants into different food products to achieve health benefits. Though providing similar benefits, products may differ in the acceptability and bioavailability of the target antioxidants. Snack bars enriched with cocoa flavanol were found to lower total and LDL cholesterol levels (Polagruto *et al.*, 2006). The study participants were given 2 servings per day for six weeks. Similarly, milk fortified with phytosterols was found to reduce cholesterol levels in healthy and

hypercholesterolemic subjects after only 15 days (Goncalves *et al.*, 2007). High blood cholesterol levels can lead to atherosclerosis and an increased risk of thrombosis (Tapiero *et al.*, 2003).

Some studies have substituted ingredients with health beneficial properties in place of ingredients that have little to no value added. Fish oil used instead of margarine in bread, was found to significantly increase the quantity of long-chain omega-3 fatty acids in blood plasma (Saldeen *et al.*, 1998). A sensory analysis was performed to determine the consumer acceptability of the bread product. Very few people could detect an aroma of fish in the bread baked with fish oil.

Functional components were incorporated into bread to yield prebiotic and pre-aox bread (Seidel *et al.*, 2007). The prebiotic bread contained inulin, linseed and soya fibre, while the prebiotic antioxidant bread (pre-aox-bread), also contained green tea powder, herbs and tomato paste. All caloric intake and daily intake of bread was kept the same. The ferric reducing ability of plasma increased after treatment with the antioxidant prebiotic bread in nonsmokers but showed no change in smokers. Carotenoids were increased with the prebiotic antioxidant bread, and ICAM-1 (a stress marker) decreased after consuming the prebiotic bread. This study shows that a bread product, even after the baking process could potentially have health-beneficial effects for the consumer.

Summary

Functional foods, specifically a bread model, may serve as a dietary delivery source for many health-beneficial compounds. However, different additives affect the final product along with variables, such as baking temperatures and time. This may be a

challenge in developing a functional bread model with grape seed-based nutraceutical ingredients. Additional research is required to investigate viability of a functional bread model containing grape seed by-products, the health beneficial effects of such a product, and possible side effects to promote their application in improving human health.

Chapter 3: Chemical Composition and Health Properties of the Selected Cold-Pressed Grape Seed Flours and Oils

Grape (*Vitis vinifera*) seed flours and oils may function as dietary sources of antioxidants. In this study, chemical tests were performed to quantify the health beneficial factors of Ruby Red, Norton, Chardonnay, Concord, and White grape seed oils and flours. Flours were extracted with three solvents (70% ethanol, 50% acetone, or 70% acetone with 0.5% acetic acid), while oils were extracted with 90% methanol. Extracts were then tested for their total phenolic contents and scavenging ability against peroxy, hydroxyl and DPPH radicals, while oils were tested for carotenoid and tocopherol contents, fatty acid composition, and oxidative stability. Lastly, the antiproliferative effects of the flour and oil extracts were tested on HT-29 human colon cancer cells. Based on the resulting data, the cold-pressed Chardonnay grape seed flour was identified as a potential functional food ingredient deserving further analysis.

Introduction

The human body functions through carefully monitored oxidation-reduction reactions, both inside and outside cells, that are used to maintain metabolism and general health. Oxidative stress can shift the delicate balance of oxidation and reduction, causing undesirable elevated levels of free radicals in the body (Hennig *et al.*, 2007). Free radicals are strong oxidative chemicals that are found naturally in vivo as products of metabolism and are used advantageously in certain biological functions, including an important role in the ability of immune system cells to protect against invading species. However, sustained

elevated levels of free radicals can lead to uncontrolled and unwanted reactions between free radicals and vital macromolecules in cells. For instance, free radicals can attack and significantly alter the structure of DNA, which can lead to the development of cancerous cells (Seifried *et al.*, 2007). There are many other mechanisms by which oxidative stress can contribute to incidences of chronic illness. Antioxidants can alleviate oxidative stress in the body by reducing hyper-reactive oxidative species, thereby restoring the chemical balance in the body (Seifried *et al.*, 2007).

It has been shown that grapes contain antioxidant compounds that have specific health beneficial properties (Prior & Gu, 2005; Karthikeyan *et al.*, 2007). Grapes contain phenolic antioxidants, including resveratrol, anthocyanins, and flavanols, among others (Kim *et al.*, 2006; Choi & Lee, 2008). Consumption of these compounds is believed to reduce the risk of certain human illnesses, including cardiovascular disease and cancer (Fan & Lou, 2004; Zern *et al.*, 2005). The seeds of the grape are rich in the phenolic compounds that are believed to contribute to these health benefits.

The present study was conducted to investigate the chemical composition, free radical scavenging capacity, and anti-proliferative activities of the cold-pressed seed flours and oils of five different grape (*Vitis vinifera*) varieties: Chardonnay, Concord, Norton, White, and Ruby Red. The data obtained from this study will be used to identify varieties high in health-beneficial phytochemicals with potential for use as functional food ingredients.

Materials and Methods

Materials and Chemicals

Cold-pressed flours and oils from the five varieties of grape seeds were provided by Botanical Oil Innovations Inc. (Spooner, WI). These seed flours were composed of the solid residues remaining after cold-pressed seed oil production and milling. Gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), Folin-Ciocalteu (FC) reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and α -tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA), and 30% ACS grade H₂O₂ was purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was manufactured by Cayman Chemical Co. (Ann Arbor, MI) and used in all experiments. Cell culture media components were obtained from Invitrogen (Carlsbad, CA), and an ATP-Lite 1step Luminescence Assay System was obtained from Perkin-Elmer (Waltham, MA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Lipid Extraction

Grape seed flour was ground in a standard household coffee grinder to a consistency able to pass through a 40-mesh sieve. Hexane was used to de-fat the sample at a ratio of 1 g flour to 9 mL hexane. The samples were centrifuged for 10 minutes at 3000 rpm. Vacuum filtration was used to collect the samples and remaining hexane was evaporated in a nitrogen evaporator.

Antioxidant Extraction

The grape seed flour samples remaining after hexane extraction were extracted with three different solvents: 70% ethanol, 50% acetone, and 70% acetone with 0.5% (v/v) acetic acid. Samples were extracted in a ratio of 1 g to 10 mL (Parry *et al.*, 2006). The samples were vortexed three times for 30 seconds each. The samples were left on a 3-D rotator for 18 hours in the dark, and centrifuged prior to decanting of the supernatant for testing.

Grape seed oil samples were extracted with 90% methanol in a ratio of 1 g to 9 mL. The samples were vortexed for 4 minutes and placed in the centrifuge for 5 minutes at 1,000 x g. The supernatant was collected, and the oil sample was extracted with 90% methanol two more times, and the supernatants were combined.

Fatty Acid Composition

Prior to gas chromatographic analysis, volatile derivatives of fatty acids were prepared by saponification followed by methylation. This conversion of free fatty acids to fatty acid methyl esters (FAME) was performed using a previously outlined laboratory procedure (Yu *et al.*, 2002). The FAME samples were subjected to GC analysis to identify the fatty acid composition of the grape seed oil samples, using a Shimadzu GC-2010 with an FID, an AOC-20i injector, and an AOC-20S autosampler (Columbia, MD). Helium was used as the carrier gas at a flow rate of 12.2 mL/min through a fused silica capillary column SPTM-2380 (30 m × 0.25 mm with a 0.25 µm film thickness). Injection volume was 1 µL with a split ratio of 10:1. Oven temperature was initially 136 °C, increased by 6 °C/min until 184 °C where it was held for 3 min, then increased again by 6 °C/min to a

final temperature of 226 °C. The relative fatty acid profile of each variety was determined by comparing sample peaks in triplicate to that of known standards in order to identify the components.

Carotenoid/Tocopherol Composition

The grape seed oil for high performance liquid chromatography (HPLC) analysis of carotenoids and tocopherols were prepared by saponification according to a method by Kurilich & Juvik (1999) with modifications. One mL of ethanol with 0.1% BHT was added to 3 mL of oil extract. The samples were placed in an 85 °C water bath for 5 minutes, after which 2 mL of 2 M KOH was added. The samples were vortexed and returned to the water bath for 10 minutes for saponification. Immediately after saponification, the test tubes were placed in an ice bath, and 1.5 mL of 3 M NaCl and 1.5 mL of methyl *tert*-butyl ether (MTBE) were added to each sample. Samples were vortexed and centrifuged at 700 g's for 10 minutes. The supernatant was decanted to a new tube. The MTBE wash was repeated twice more, and the supernatants were combined. Deionized water was then used to wash the combined supernatants. The MTBE was evaporated and the residue was redissolved with 1 mL of MTBE (Kurilich & Juvik, 1999).

HPLC analysis was completed with the prepared samples in triplicate to determine carotenoid and tocopherol composition using a Shimadzu LC-20AD model with SPD-20A UV detector (Columbia, MD). The wavelength of the UV detector was set at 450 nm. Mobile phase A was methanol/MTBE/water (81:15:4, v/v/v) and mobile phase B was methanol/MTBE (9:91 v/v). The separation was achieved using a linear gradient of 100% of A to 50% A and 50% B in 45 min, followed by 100% B for 10 min to wash the column,

and 100% A for 5 min to re-equilibrate prior to the next injection. The flow rate was 1.0 mL/min with a column temperature of 25 °C and an injection volume of 30 µL.

Carotenoids and tocopherols were quantified using comparisons of HPLC retention time and peak area of known concentrations of purchased standards.

Total Phenolic Content

Folin-Ciocalteu (FC) reagent was used to determine the total phenolic content (TPC) of the grape seed flour and oil extracts following a previously described laboratory procedure (Yu *et al.*, 2002). A 50 µL sample of each of the grape seed flour and oil extracts was mixed with 250 µL FC reagent, 750 µL 20% sodium carbonate, and 3 mL of Ultrapure water. After reacting for 2 h at ambient temperature, absorbance was read at 765 nm on a Thermo Spectronic Genesys spectrophotometer (Waltham, MA). A separate standard curve was created for each extraction solvent using gallic acid. Results were reported as mg gallic acid equivalents per g of grape seed flour or oil, and were measured in triplicate for each sample. Sample extracts were diluted in extraction solvent as necessary to fall within the range of the standard curve.

Oxidative Stability Index (OSI)

OSI values were determined using a 743 Rancimat Metrohm (Herisau, Switzerland). The oxidation reaction was carried out at 86 °C with an air flow rate of 7 L/h, and 60 mL of deionized water in each of the measuring vessels. Samples were tested in 6 mL aliquots of the grape seed oil. Commercial canola oil was used as the control.

Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC values were determined for grape seed flour extracts following a previously described protocol (Moore *et al.*, 2005). Trolox standards were prepared in appropriate solvents matching the grape seed flour extract being tested. All other reagents were prepared in a 75 mM sodium phosphate buffer (pH 7.4). A 225 μL aliquot of freshly made 8.16×10^{-8} M fluorescein solution was mixed with 30 μL sample, standard or blank in a black 96-well plate and pre-heated at 37 °C for 20 minutes. After pre-heating, 25 μL of freshly made 0.36 M AAPH was mixed into each well, and the fluorescence of the reaction mixture was measured using a Victor³ multilabel plate reader (Perkin-Elmer, Turku, Finland) once every two minutes for two hours at 37 °C, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Sample trolox equivalents (TE) were estimated based on the area-under-the-curve (AUC) method used by Ou and colleagues (2001). Results were reported as μmol of TE per g of defatted, cold-pressed grape seed flour, and were measured in triplicate for each sample.

Hydroxyl Radical Scavenging Capacity (HOSC) Estimation

The HOSC assay was also conducted using fluorescein as the fluorescent probe and a Victor³ multilabel plate reader (Perkin-Elmer, Turku, Finland) according to a previously reported laboratory protocol (Moore *et al.*, 2006). In brief, 30 μL of sample standard or blank was reacted with 170 μL of 9.28×10^{-8} M fluorescein, 40 μL of 0.1990 M H_2O_2 , and 60 μL of 3.43 M FeCl_3 in black 96-well microplates. The fluorescence of the reaction mixture was recorded approximately once every 260 seconds for up to seven hours at ambient temperature, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Standards were prepared in appropriate solvents matching that of

the flour samples being tested. Fresh 9.28×10^{-8} M fluorescein solution was prepared for each assay from stock solution and 75 mM sodium phosphate buffer (pH 7.4). Trolox equivalents (TE) of each sample were calculated following the same AUC method used for the ORAC TE estimation (Moore *et al.*, 2006). Results were reported as micromoles of TE per g of defatted, cold-pressed grape seed flour, and were measured in triplicate in each sample.

DPPH Scavenging Activity

Free radical scavenging activity was determined according to a previously reported procedure by Yu and colleagues utilizing stable 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) radicals (2003). The reaction consisted of an equal mixture of freshly prepared 100 μ M DPPH[•] solution and grape seed extract, primed to a total volume of 200 μ L in a clear, flat-bottomed 96-well plate. The absorbance was measured at 515 nm every 1.5 minutes for 40 minutes and compared against a standard curve consisting of six dilutions of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) between 10 μ M to 35 μ M using solvent as the blank. Measurements were taken using a Victor³ multilabel plate reader (Perkin-Elmer, Turku, Finland). Results are presented as μ mol TE/ g flour or oil.

Anti-proliferative Activity against HT-29 Cells

The anti-proliferative test was adopted from a previously described experiment (Wang *et al.*, 2007). The HT-29 human colorectal adenocarcinoma cell line was cultured in a humidified incubator at 37 °C and 5% CO₂. The cell culture media contained McCoy's 5A media, 10% fetal bovine serum, and 1% antibiotic/antimycotic solution. Cells were grown to 95% confluence and then plated at 2,500 cells/well in a black 96-well

viewplate for experiment. After 24 h, the growth medium was replaced with 100 μ L of either the control or treatment medium. An ATP-Lite 1step Luminescence Assay System for Perkin-Elmer was used to determine the amount of cell proliferation prior to treatment and at 4, 24, 48, 72, and 96 h after the initial treatment. Plates were permitted to equilibrate to room temperature for a period of 30 mins before the reading was taken. Immediately before the reading was taken on a Victor³ multilabel plate reader, 100 μ L of reconstituted ATP-Lite 1step Luminescence was added to each well. The cell media was replaced with a fresh treatment or control solution every 24 h.

Treatment Media Preparation. To prepare the treatment solutions for anti-proliferative tests, grape seed oil extracts in 90% methanol were evaporated in the nitrogen evaporator at 40 °C until only water and extract remained. These samples were freeze-dried to remove the water. The flour extracts in 70% ethanol were also evaporated in the nitrogen evaporator at 40 °C. Dried extracts were then dissolved in DMSO. Treatment media were prepared by mixing DMSO extracts and cell growth media, which were then passed through 0.2 μ m retrograde cellulose syringe filters. Cells were treated with three different doses of grape seed flour and oils: 0.99, 2.0, and 9.9 mg grape seed part equivalent/ mL. All treatment doses contained the same amount of DMSO, including the vehicle control.

Statistical Analysis

Each assay was conducted in triplicate, with data reported in tables and figures as mean \pm standard deviation. Differences in means between samples were determined using one-way ANOVA and Tukey's post hoc analysis. Statistical analysis was performed using

SPSS software for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.05$.

Results and Discussion

Fatty Acid Composition

As shown in **Table 3.1**, cold-pressed oils from different varieties of grape seeds have similar fatty acid compositions, differing only slightly in their components. A number of fatty acids have health-promoting attributes. Linoleic acid (18:2) is found at high levels in the different grape varieties, and studies have shown that consumption of it may lead to a decreased risk of ischemic stroke (Iso *et al.*, 2002). Linoleic acid was measured in comparable quantities across the 5 types of grape seeds, ranging from 64.89 to 75.22 g/100 g oil in the grape seed oils, and Concord grape seeds were shown to contain the highest amount (**Table 3.1**). The linoleic acid data obtained falls within the range of previously demonstrated values of 50.1 to 77.8 g/100 g oil (Yu *et al.*, 2005). It was determined that each grape variety had similar oleic acid (18:1) compositions, ranging from 14.85 to 22.99 g/100 g oil, showing a slightly higher level in the Ruby Red variety (**Table 3.1**). These results show that while each of the oils obtained from the different grape varieties had slightly different fatty acid profiles, each fatty acid was present in similar overall proportions in all of the different samples. Based on fatty acid contents, no one grape seed oil stands out above the others as a better functional food ingredient.

Table 3. 1
Fatty Acid (FA) Composition of the Studied Cold-Pressed Grape Seed Flours (g/100 g oil)*

FA	Chardonnay	Concord	Norton	Ruby Red	White
16:0	7.24 ± 0.01	6.84 ± 0.01	7.62 ± 0.01	6.87 ± 0.01	7.25 ± 0.01
18:0	3.87 ± 0.01	2.79 ± 0.01	4.19 ± 0.01	4.64 ± 0.01	3.92 ± 0.01
20:0	0.19 ± 0.01	NA	0.19 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
16:1	0.09 ± 0.01	NA	0.08 ± 0.01	0.05 ± 0.01	0.08 ± 0.00
18:1	19.79 ± 0.02	14.85 ± 0.01	16.79 ± 0.00	22.99 ± 0.00	15.96 ± 0.02
18:2	68.59 ± 0.01	75.22 ± 0.04	70.71 ± 0.01	64.89 ± 0.01	72.35 ± 0.01
18:3	0.22 ± 0.01	0.29 ± 0.01	0.42 ± 0.01	0.19 ± 0.01	0.26 ± 0.01

*Values are based on triplicate readings. Data were expressed as mean ± SD (n=3). SFA: saturated fatty acids. MUFA: monounsaturated fatty acids. PUFA: polyunsaturated fatty acids. na: not analyzed.

Carotenoid/Tocopherol Composition

The carotenoid and tocopherol contents of the grape seed oils are summarized in **Figure 3.1**. The grape seed oils were analyzed for α -tocopherol and two carotenoids: lutein and β -carotene. Both carotenoids and tocopherols are antioxidants. The oils from the various grape seeds were evaluated for their α -tocopherol contents. All oil samples contained α -tocopherol, with the lowest value detected in the Concord grape seed oil at

10.65 $\mu\text{g/g}$ oil, and the highest value detected in the Ruby Red grape seed oil at 87.09 $\mu\text{g/g}$ oil. Lutein and β -carotene were also detected in all of the grape seed oils. The consumption of lutein has also been linked to an increase in pigmentation in the macular region of the retina, which decreases the risk for certain eye diseases, including light-induced retinal damage and age-related macular degeneration (Landrum & Bone, 2001). Lutein was found to be the most abundant carotenoid compound present in the grape seed oils for all five varieties. Specifically, the greatest lutein content was found in the extract of the White grape seed oil at 41.67 $\mu\text{g/g}$ (**Figure 3.1**). These results are comparable to a previous study that also reported lutein as one of the primary carotenoids found in grapes (Guedes de Pinho *et al.*, 2001). In contrast, β -carotene was found to be the least abundant of the measured lipophilic antioxidants.

The oil from all five grape seed varieties contained both carotenoid compounds, with the Norton grape seed oil sample having the highest total carotenoid content at 68.84 $\mu\text{g/g}$, followed by the oil of the White grape seed at a total carotenoid content of 60.32 $\mu\text{g/g}$. The White grape seed oils contained the greatest difference of carotenoids with 41.67 $\mu\text{g/g}$ oil of lutein and 18.65 $\mu\text{g/g}$ oil of β -carotene. The Concord grape seed oil contained the least amount of both lutein and β -carotene, at 7.39 and 4.23 $\mu\text{g/g}$ oil respectively (**Figure 3.1**).

There was no correlation between carotenoid and tocopherol contents. Grape seed oils containing higher carotenoid amounts did not necessarily correlate to higher α -tocopherol amounts. For example, Ruby Red grape seed oil was found to have the highest amount of α -tocopherol, yet contained one of the lowest amounts of carotenoid compounds. Concord grape seed oil was found to have the lowest amounts of both

carotenoid and α -tocopherol compounds. The data indicates that grape seeds of different varieties generally contain varying levels of carotenoids and α -tocopherol, but all varieties do contain at least some of both carotenoids and tocopherols. Based on carotenoid and tocopherol data, different grape seeds could be used as a functional food ingredient; the Ruby Red and Chardonnay varieties for α -tocopherol, and Norton and White varieties for carotenoids.

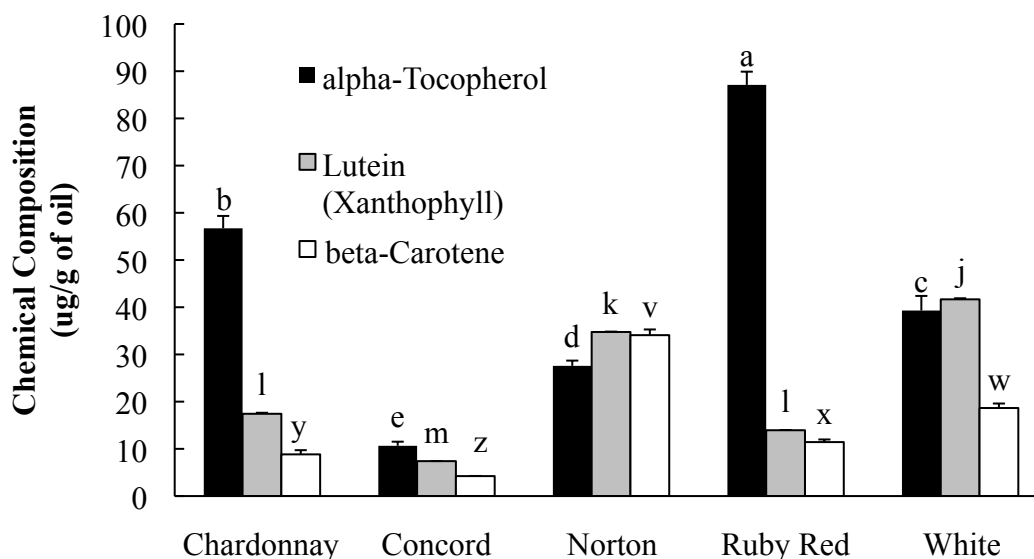


Figure 3. 1

Composition of α -tocopherol and carotenoids in grape seed oils. Values are expressed as micrograms of analyte per gram of grape seed oil. Mean and standard deviation are shown ($n = 3$). The black boxes represent α -tocopherol, grey boxes represent lutein, and white boxes represent beta-carotene. For each analyte, values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$).

Total Phenolic Content

The TPC values of each grape seed flour extract are shown in **Figure 3.2**. The TPC of the grape seed oil extracts were tested, but levels were too low to be quantified.

Chardonnay grape seed flour showed the highest level of total phenolics in all extraction solvents, followed by Ruby Red, and White varieties. Concord and Norton contained the lowest total phenolics and contained statistically the same amounts for all three solvents. Grape seed flour samples extracted with 50% acetone exhibited the highest levels of phenolics; the greatest was Chardonnay with a TPC value of 129.8 mg gallic acid equivalent (GAE)/g flour. Norton exhibited the lowest TPC value at 17.4 mg GAE/g flour when extracted with 50% acetone (**Figure 3.2**).

Extraction with different solvents yielded differing estimations of phenolics. Chardonnay seed flour extracted with 50% acetone, as discussed above, had significantly greater level of phenolics (129.8 mg GAE/g flour) than Chardonnay seed flour extracted with 0.5% acetic acid in 70% acetone (105.9 mg GAE/g flour). The solvent, which consistently extracted the lowest levels of phenolics, was 70% ethanol, with a TPC value of 74.5 mg GAE/g flour for Chardonnay seeds. Varying levels of phenolics with different extraction solvents indicate 0.5% acetic acid and 70% ethanol are not extracting phenolics present as well as 50% acetone (**Figure 3.2**).

White or light grape varieties have been shown previously to have a higher total phenolic content than red or purple grape varieties (Pastrana-Bonilla *et al.*, 2003). Chardonnay seed flour (a white grape) exhibited a TPC value of 186.3 mg GAE/g flour, which was higher than all other fruit seeds in the study (Parry *et al.*, 2006). Chardonnay

seed flours also contained the highest TPC of the 5 grape varieties in the present study. Grape seed oils are known to have low levels of phenolics, ranging between 0.06-0.1 mg GAE/g seed oil (Bail *et al.*, 2008), which may explain why their levels were undetectable using the method of the current study. Yilmaz and Toledo (2006) showed the total phenolic content of grape seeds was affected by extraction solvent. In their study, extraction with 50% acetone solution exhibited a greater TPC value than extraction with 70% methanol solution; a 0.5% acetic acid solution was not compared (Yilmaz & Toledo, 2006). Variations in phenolic content of grape seed flours can also result from variations in genetics and growing conditions. Based on TPC data, Chardonnay seed flour was a frontrunner candidate as a functional food ingredient.

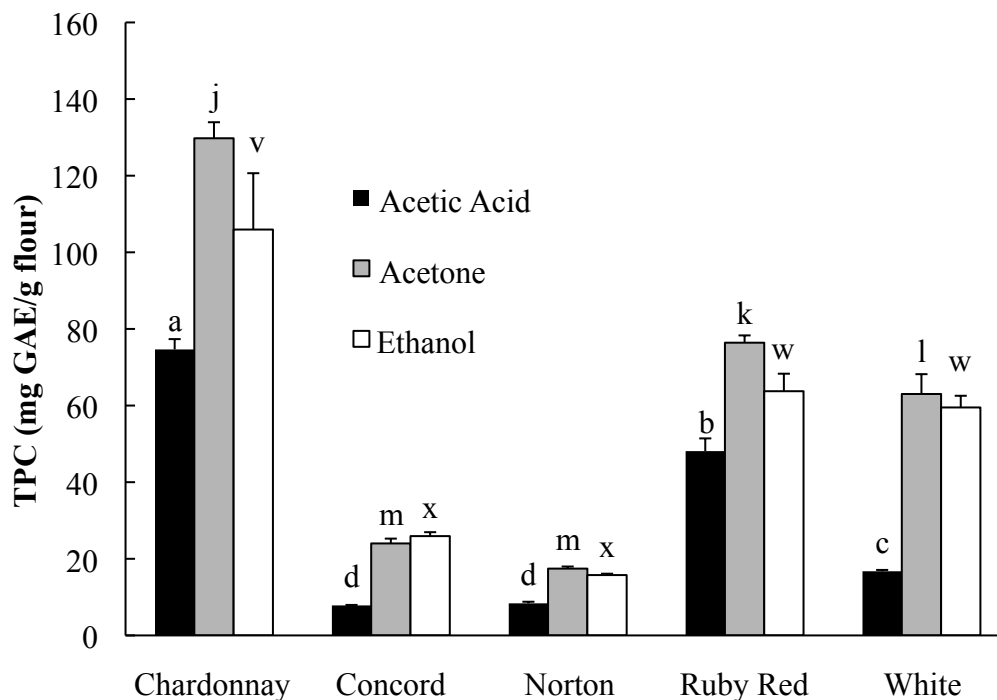


Figure 3. 2

Total Phenolic Contents of Grape Seed Flours. Values were determined spectrophotometrically using Folin-Ciocalteu reagent. Gallic acid was used as the standard. Results shown as mg of gallic acid equivalents (GAE) per g grapeseed flour. The black columns represent an extraction solvent of 70% acetone with 0.5% (v/v) acetic acid, the grey columns represent 50% acetone, and the white represents 70% ethanol. For each solvent listed, values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$)

Oxidative Stability Index (OSI)

Grape seed oil from the Norton, Ruby Red, White, and Concord varieties showed a significantly increased Oxidative Stability Index (OSI) compared to the canola oil control. OSI is a measurement taken of oils under accelerated oxidation conditions—it is the time at which an oil experiences the maximum change in the rate of oxidation. A higher OSI value (i.e., a longer time to reach the maximum change in the rate of oxidation) is

indicative of a better shelf life. It is an indicator for the durability of the oil during storage (American Oil Chemist Society (AOCS), n.d.). This is an important factor when considering the shelf life of our product. As seen in **Figure 3.3**, the Norton and Ruby Red varieties exhibited the highest OSI. To make realistic comparisons, canola oil was used as a comparative control, as this oil is commonly used.

OSI is determined by a variety of properties inherent to a food, including presence of polyunsaturated fatty acids (PUFA). OSI is inversely related to oil's PUFA content, as unsaturated fats are more easily oxidized. OSI is also determined by the tocopherol content of the oil, and in general more tocopherol is correlated with a higher OSI value, though the relationship is not linear (Tappel, 1998). The data indicated that Ruby Red oil has the lowest PUFA content and high tocopherol content, we observed a relatively high OSI value for Ruby Red grape seed oil. Norton, which had a low PUFA content and relatively low tocopherol content, had a high OSI value. The OSI value for the Chardonnay variety was unexpectedly low, falling below that of canola oil despite its low PUFA content (which was lower than that of the Norton variety) and high tocopherol content.

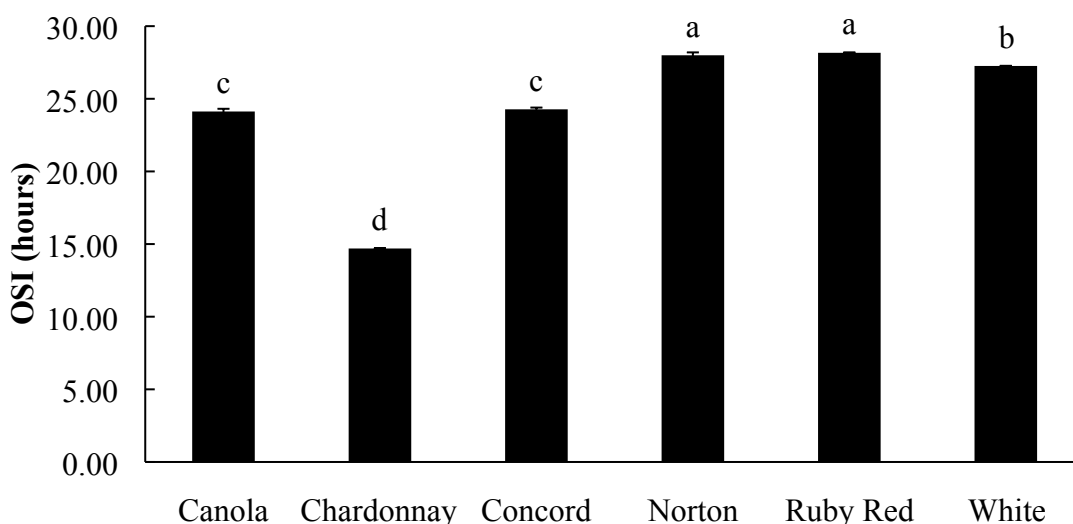


Figure 3. 3

Oxidative Stability Index (OSI) of grape seed oils. The OSI for the cold-pressed grape seed oils of each variety and canola oil control were measured and the hours until oxidation are recorded. For each analyte

Antioxidant Properties

All grape seed flour extracts exhibited scavenging capacities against peroxy (ORAC) and hydroxyl (HOSC) radicals. Chardonnay grape seed flour was consistently present in the highest statistical group for antioxidant activity in every solvent tested ($P \leq 0.05$) (**Figures 3.4 & 3.5**). For HOSC in 70% ethanol and 50% acetone, Chardonnay exhibited the highest radical scavenging values of 2,212 and 2,774 $\mu\text{mol trolox equivalents (TE)}/\text{g}$ defatted seed flour, respectively (**Figure 3.4**). For ORAC 70% ethanol and 0.5% acetic acid in 70% acetone, Chardonnay exhibited the highest TE values of 1,429 and 1,131 $\mu\text{mol TE}/\text{g}$ defatted seed flour, respectively (**Figure 3.5**). In the 50% acetone samples, Ruby Red exhibited a value of 2013 $\mu\text{mol TE}/\text{g}$ defatted seed flour in

comparison to 1,864 $\mu\text{mol TE/g}$ for Chardonnay; however, the two were not statistically different at the $P \leq 0.05$ significance level. ORAC and HOSC values of Chardonnay found are higher than that of previously reported values for Chardonnay seed flour (Parry *et al.*, 2006; Yilmaz & Toledo, 2006), but are similar to ORAC values reported for seeds of Turkish grape varieties (Bozan *et al.*, 2008).

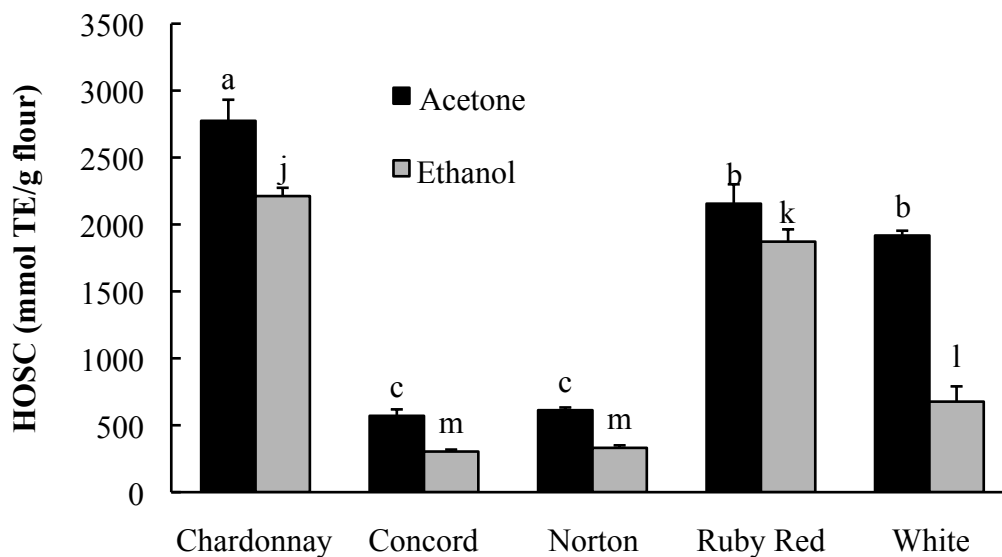


Figure 3. 4

Hydroxyl radical scavenging capacity of the cold-pressed grape seed flour extracts.

Values were measured against a trolox standard and expressed as μmol of trolox equivalents (TE) per g of defatted seed flour. Mean + SD values are shown ($n = 3$). The black column represents an extraction solvent of 50% acetone, and the grey represents an extraction solvent of 70% ethanol. For each solvent listed, values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$)

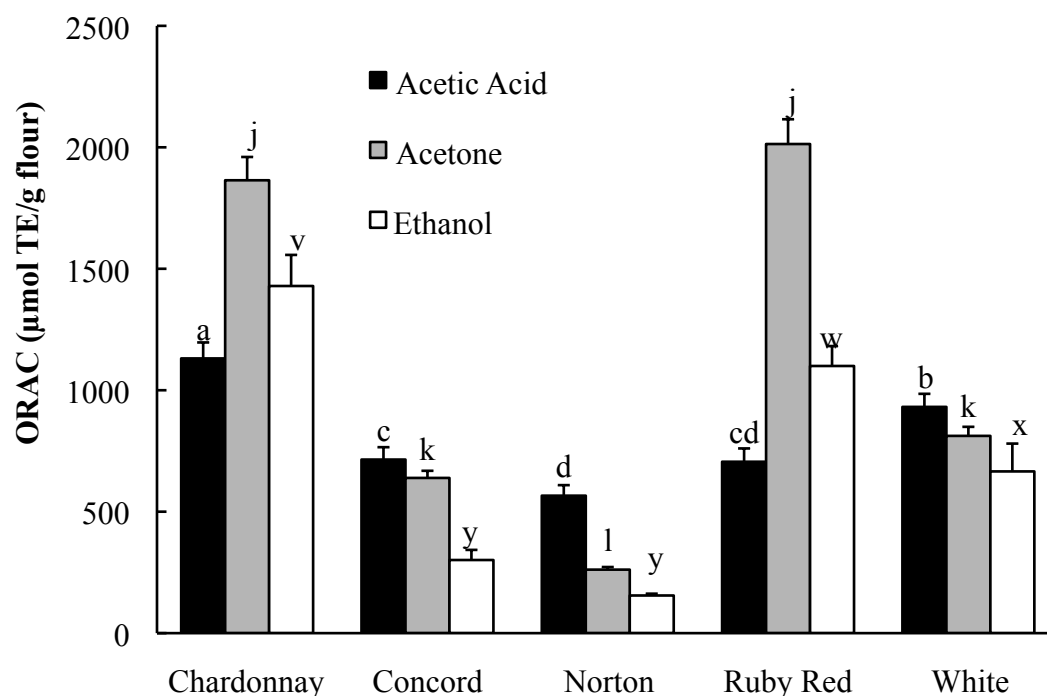


Figure 3. 5

Oxygen radical scavenging capacity of cold-pressed grape seed flour extracts. Values were measured against a trolox standard and expressed as μmol of trolox equivalents (TE) per g of defatted seed flour. Mean + SD values are shown ($n = 3$). The black columns represent an extraction solvent of 70% acetone with 0.5% (v/v) acetic acid, the white columns represent an extraction solvent 50% acetone, and the grid represents an extraction solvent 70% ethanol. For each solvent listed, values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$)

All grape seed flour extracts also exhibited DPPH \cdot Scavenging Activity. As seen in **Figure 3.6A**, Chardonnay extract exhibited the highest activity in all three solvents with 8.23 mmol (0.5% acetic acid), 9.21 mmol (50% acetone), and 6.49 mmol TE/g of flour (70% ethanol), followed by Ruby Red and White, then Concord and Norton. Activities exhibited by Ruby Red and White extracts were similar to each other. Concord and Norton had the lowest activity, and were similar to each other. The 50% acetone and 0.5% acetic

acid extracts were also comparable to each other, while the ethanol extracts exhibited significantly lower DPPH \cdot scavenging activity for all grape varieties.

Of the grape seed oil extracts in methanol, Concord exhibited the highest activity with 11.08 μ M TE scavenged per g of oil (**Figure 3.6B**). The remaining grape seed oil extracts were statistically similar, ranging from 3.09 to 5.92 μ M TE/g of oil.

The DPPH \cdot scavenging activity for Chardonnay grape seed flour has previously been identified to be 1.51 mmol TE/g of flour in 50% acetone (Parry *et al.*, 2006). The approximate three to five fold difference can partly be attributed to the specificity of cultivars (De Beer *et al.*, 2003), as well as potentially different growing conditions. Other reported DPPH \cdot scavenging activity includes the activity of Norton and Concord grape skins, at 0.9 mmol of TE/g and 0.8 mmol of TE/g, respectively (Munoz-Espada *et al.*, 2004). Grape seed flour extract in acetic acid for Norton (2.51 mmol TE/g) and Concord (2.61 mmol TE/g) were greater than that of grape seed skins while the respective grape seed flour extracts from ethanol were fairly similar. Overall, the data have implication in improving extraction of antioxidants from natural sources and the potential health properties of different types of grapes and their by-products, specifically the Chardonnay variety, as a food ingredient.

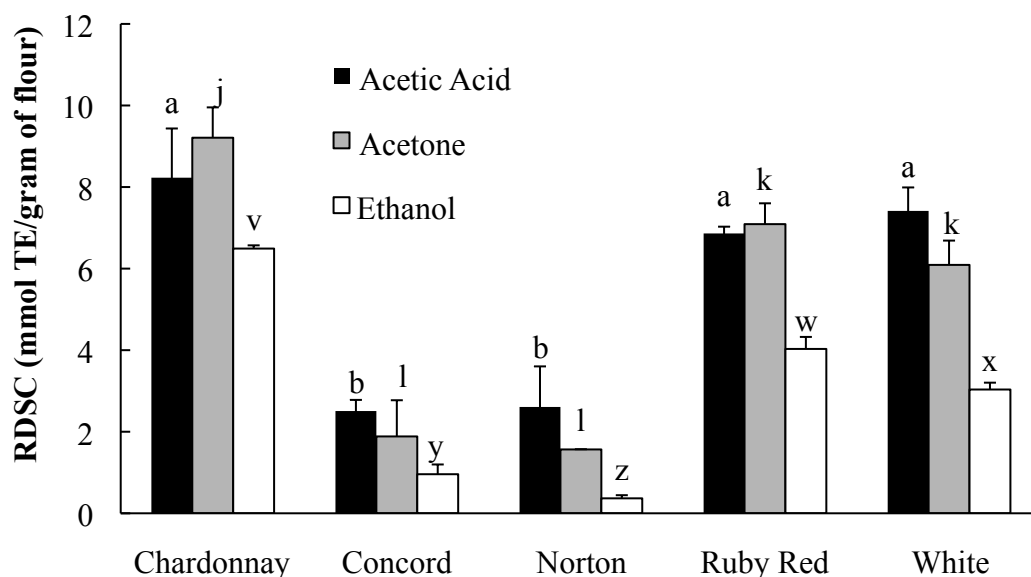
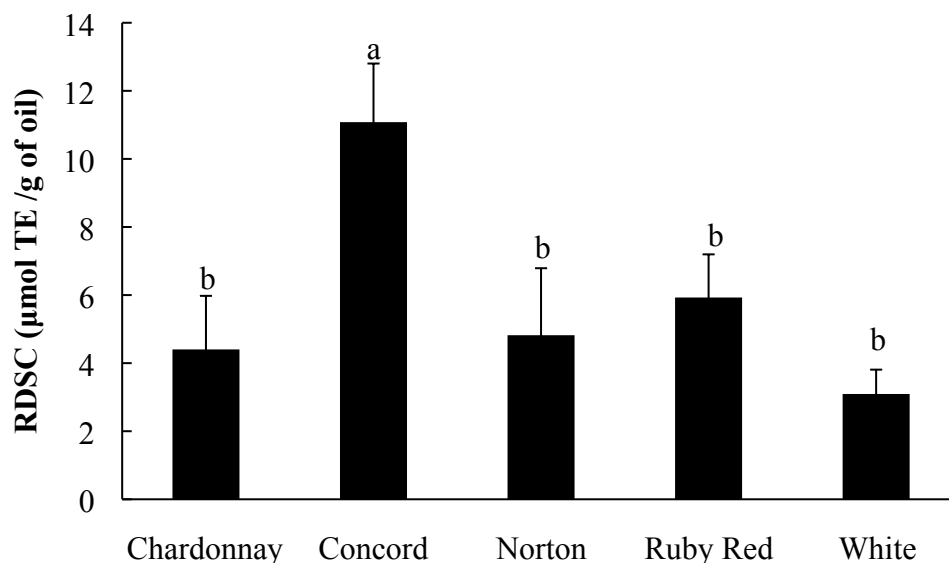


Figure 3. 6A

DPPH[•] scavenging activity of grape seed flour. Samples were evaluated for direct scavenging activity of DPPH radicals and compared to a trolox standard. Results are shown as mean \pm SD in units of millimoles of trolox equivalents (TE) per gram of defatted grape seed flour. The black columns represent 70% acetone with 0.5% (v/v) acetic acid, the white columns represent 50% acetone, and the grey represents 70% ethanol.



Figure

3.6B.

DPPH Radical Scavenging capacity of grape seed oils. Samples were evaluated for direct scavenging activity of DPPH radicals and compared to a trolox standard. Results are shown as mean \pm SD in units of micromoles of TE/gram of grape seed oil. The black columns represent 90% methanol. Columns in a series marked by the same letter are not statistically different ($n = 3$; $P \leq 0.05$).

Anti-proliferative Activity against HT-29 Cells

The results of the anti-proliferation HT-29 cell study show that the variety and concentration of grape seed flour and oil dictate the amount of cells that remain after treatment over time. The flours and oils, in three different concentrations, were compared with a vehicle control containing the same concentration of DMSO and were treated in the same manner. In each of the flour and oil samples, the high concentration (9.9 mg grape seed part equivalent/mL) of all of the grape treatments resulted in the greatest amount of cell inhibition after 96 h. For the Concord flour and oil, Chardonnay flour, Ruby flour, and White flour and oil, the number of cells actually decreased ($P < 0.05$) over the entire 96 h in the high concentration of treatment. This shows that these types of extract and variety

of grapes give a high value of anti-proliferative effects for these cancer cells. The vehicle control was used to show the exponential growth of the cancer cells given the media and sera combination used, and was used for comparison of the growth of treated cells. The high concentration treatments, 9.9 mg equivalence/mL, of White, Ruby, and Chardonnay flours virtually eliminated the cancer cells within those samples after 48 hours, showing an extremely high level of effectiveness. These varieties also showed decreased growth of cancer cells in the low (0.99 mg grape seed part equivalent/mL) and medium (2.0 mg grape seed part equivalent/mL) treatment concentration levels when compared to the vehicle solution. The oils were less effective in slowing the growth of the cancer cells, but were still able to decrease the amount of cell growth when compared to the vehicle control. As seen in **Figure 3.7**, the flours of White, Ruby, and Chardonnay grapes were the most effective and statistically significant in the anti-proliferation assay. This tentatively supports previous findings where treatments of 3 mg grape seed part equivalents/mL and 6 mg grape seed part equivalents/mL of Chardonnay grape seed flour extract was found to have nearly eliminated HT-29 cancer cells after 24 hours of treatment (Parry *et al.*, 2006). The treatments in the present study were not as dramatically effective, but this still confirms the clear trend that grape seed extract inhibits the growth of these cells. To evaluate the potential utilization of these grape seed flours and oils in cancer prevention, it is necessary to complete more evaluative tests on other cancer cell lines, as well as normal cell lines. However, the results here suggest that Chardonnay, Ruby Red, and White are the most bio-active and are therefore have the most as potential functional food ingredients.

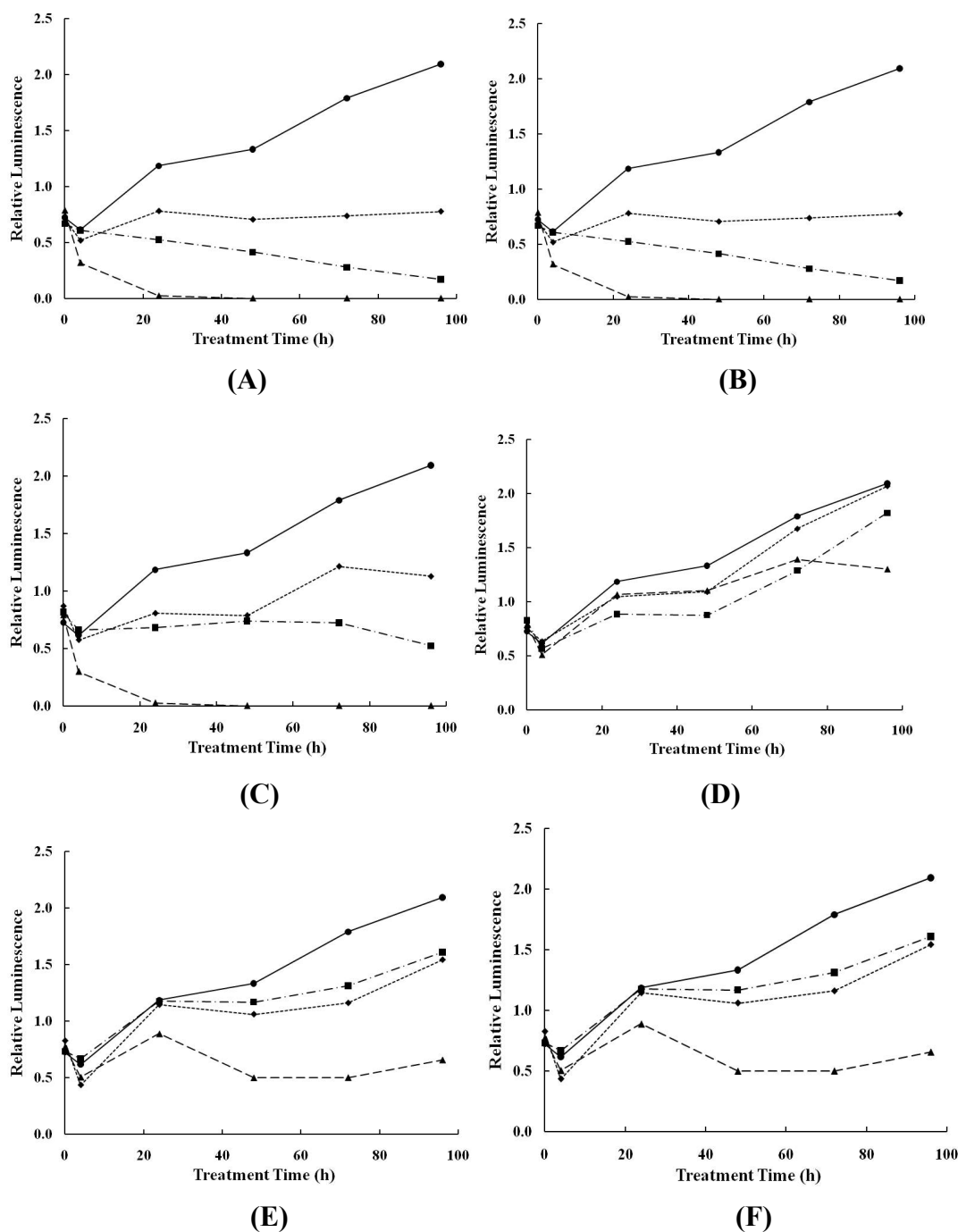


Figure 3.7

Time and Dose Effects of Grape Seed Flour and Oil Extracts on HT-29 Cell Proliferation.

Relative luminescence is proportionate to the number of viable cells.

Values are based on triplicate tests, with mean values show (n = 3). (A) Chardonnay Flour

(B) Chardonnay Oil (C) Ruby Red Flour (D) Ruby Red Oil (E) White Flour (F) White Oil.

The diamond represents the low treatment of 0.99 mg grape seed part equivalents/mL, the

square represents the medium treatment of 2.0 mg grape seed part equivalents/mL and the triangle represents the high treatment of 9.9 mg grape seed part equivalents/mL. Vehicle is represented by a circle.

Conclusion

About 66 million tons of grapes were produced world wide in 2007; of these fresh grapes, 86.6% were further processed into wine, jams, and grape juice (Maier *et al.*, 2009). Grape seeds are a waste product of the juice and wine industry, which generates large quantities of waste (5–9 million tons per year, worldwide), which can seep into the ground and increase the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD). Changing these characteristics of the natural environment can have detrimental effects on the flora and fauna of the waste discharge zones. Finding another use for grape seeds could help to ameliorate this environmental problem (Bonilla *et al.*, 1999; Schieber *et al.*, 2001; Louli *et al.*, 2004). The results from this study indicate that grape seed flours and oils may serve as dietary sources of natural antioxidants that contain anti-proliferative properties, particularly Chardonnay grape seed flour. Additional research is necessary to further analyze the effects of food formulation, processing, and storage on the availability of the beneficial properties.

Chapter 4: Chemical Composition and Health Properties of Whole Soft Wheat Bread Enriched with Cold-Pressed Chardonnay Grape Seed Flour and Oil

The present study was conducted to investigate bread baked with the grape seed flour and oil at different times and temperatures, for their chemical composition, free radical scavenging capacities, and anti-proliferative activity against cancer cells. The data obtained from this study was used to determine the refined time and temperature that a functional food, such as bread, that has been incorporated with the grape seed components can still exhibit the maximum amount of available health beneficial properties.

Introduction

The human body functions by maintaining metabolism through carefully monitored oxidation-reduction reactions. Oxidative stress can shift the balance of these reactions, causing elevated levels of free radicals in the body (Hennig *et al.*, 2007). Chronically elevated levels of free radicals can lead to uncontrolled reactions with vital macromolecules (Seifried *et al.*, 2007). Grapes and grape seeds contain phenolic antioxidants that have been shown to contain health-beneficial properties by mitigating oxidative stress in the body caused by hyper-reactive oxidative species (Kim *et al.*, 2006; Seifried *et al.*, 2007; Choi & Lee 2008). However, 67% of American adults consume less than two servings of fruit a day (Blanck *et al.*, 2005). Grapes as fruits are not always available, affordable, or a practical choice for consumers. A functional food model allows

consumers to obtain additional antioxidants in a less perishable, economical product that is readily available. Bread has been previously chosen as the functional food model for other fruit additives due to its ease of preparation and status as a common food product (Fan *et al.*, 2006).

During processing, specifically baking, changes in chemical composition and food matrix alter the composition of antioxidants in a functional food model (Delgado-Andrade *et al.*, 2010). Availability of these antioxidants may be affected by variation of food processing conditions such as baking time and temperature, either positively or negatively as new compounds are formed or others are released from the food matrix (Moore *et al.*, 2009). Original concentrations in raw ingredients and changes during incorporation into a functional food also affect bioavailability of factors critical for health-beneficial effects. The changes may be a result of chemical and biochemical reactions during storage and ingredient processing and food preparation, as well as interactions with other components during digestion (Kim *et al.*, 2006). Bioavailability can vary drastically during digestion. In one case following both oral and gastric steps the amount of soluble antioxidants increased by approximately 17 fold while insoluble antioxidants decreased by approximately 10 fold (Delgado-Andrade *et al.*, 2010). Results of efficacy trials involving bioavailable antioxidants also fluctuate wildly. Studies have shown in rats that antioxidants administered with a dry diet can predictably inhibit development of carcinogen induced cancer while antioxidant extracts administered with sesame oil did not (Kim *et al.*, 2004).

The present study was conducted to assess the availability (*in vitro*) of antioxidants, radical scavenging capacity, and efficacy of a grape seed bread functional

food model made with cold-pressed Chardonnay grape seed flour and oil. Data from this study will be used to determine optimal baking time and temperature as well as viability of grape seed bread as a source of antioxidants.

Materials and Methods

Materials and Chemicals

Cold-pressed chardonnay grape seed flour and oil were provided by Botanical Oil Innovations Inc. (Spooner, WI). Gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), Folin-Ciocalteu (FC) reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β -carotene, and α -tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA), and 30% ACS grade H₂O₂ was purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was manufactured by Cayman Chemical Co. (Ann Arbor, MI) and used in all experiments. Cell culture media components were obtained from Invitrogen (Carlsbad, CA), and an ATP-Lite 1step Luminescence Assay System was obtained from Perkin-Elmer (Waltham, MA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Baking Process

The bread samples were baked using a standard recipe and food-grade ingredients. First 118 g lukewarm water at approximately 27 °C was added to 4 g sugar and 6 g yeast. This mixture was allowed to sit for ten minutes to proof the yeast. Meanwhile, the dry

ingredients were mixed separately; this included 4 g salt, 33 g sugar, and 88 g soft whole wheat flour. For the control product, 88 g of bread flour was also added to this mixture. For the grape seed product, 44 g bread flour and 44 g grape seed flour was added to the mixture. In addition to the unbaked control samples, control and grape seed bread samples were baked for each of the following conditions: 163 °C for 30 minutes, 191 °C for 20 minutes, 191 °C for 30 minutes, 191 °C for 40 minutes, and 218 °C for 30 minutes. Each sample was prepared and baked in triplicate.

Lipid Extraction

Freeze-dried bread samples with and without grape seed flour were ground in a standard household coffee grinder to pass through a 40 mesh sieve. Hexane was used to extract the fat from the bread samples at 1g sample to 2 mL hexane ratio at ambient temperature for 5 minutes. The samples were centrifuged for 10 minutes at 3000 rpm. The hexane extraction was collected and stored at -16 °C for further analysis.

Antioxidant Extraction

The defatted bread samples were extracted with 50% acetone at 1 g of sample to 4 mL solvent ratio. They were vortexed three times for 30 seconds each and then left in a 3-D rotator for 18 hours in the dark. The supernatants were decanted and stored at -16 °C until further analysis.

Lutein/Tocopherol Composition

Sample Preparation. In order to prepare the lipid bread extracts for high performance liquid chromatography (HPLC) analysis, 1 mL of methyl tertiary butyl ether (MTBE) was added to a test tube containing 3 mL of the extract. The test tube was sealed and placed in a sonicator for 6 minutes. 0.2 mL of the sample was extracted from the test tube, and filtered through a 0.45 µm filter. The preceding steps were completed for all of the 36 samples (Darnoko *et al.*, 2000).

HPLC Analysis. The HPLC system used to determine the lutein and tocopherol composition of the bread samples was a Shimadzu LC-20AD model SPD-20A UV detector EZstart system with a C-30 column with a length of 250 mm and a particle size of 5 µm. The wavelength for the detection was set at 450 nm for luteins and 295 nm for tocopherols. Mobile phase A was methanol/MTBE/water (81:15:4, v/v/v) and mobile phase B was methanol/MTBE (9:91, v/v). The gradient elution was as followed: 100% A to 77.8% A/ 22.2% B from 0 to 20 minutes, 100% B from 20 to 30 minutes, and re-equilibration of the column at initial gradient conditions from 30 to 40 minutes. The flow rate was set at 1.0 mL/min, and 30 µL of each sample was injected into the HPLC system using the auto sampler. Identification and quantification of luteins and tocopherols were based on comparisons between HPLC retention time, and area under the curve of the sample peak with that of the standards (Darnoko *et al.*, 2000).

Total Phenolic Content

Folin-Ciocalteu (FC) reagent was used to determine the total phenolic content (TPC) of the 50% acetone extractions of the grape seed flour enriched and control breads

following a previously described laboratory procedure (Yu *et al.*, 2002). A 50 μL sample of each of the extracted breads was diluted and mixed with 250 μL FC reagent, 750 μL 20% (m/v) sodium carbonate, and 3 mL of Ultrapure water. After reacting for 2 hours at ambient temperature, absorbance was read at 765 nm on a Thermo Spectronic Genesys spectrophotometer (Waltham, MA). The standard curve was created using different concentrations of gallic acid and results were reported as milligrams of gallic acid equivalent per gram of bread.

Oxygen Radical Absorbance Capacity

ORAC values were determined following a previously described protocol (Moore *et al.*, 2005). Trolox standards and bread extractions were diluted to appropriate amounts in 50% acetone. All other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). 225 μL of freshly made 8.16×10^{-8} M fluorescein solution was mixed with 30 μL sample, standard or blank in a 96-well plate and pre-heated at 37 °C for 20 minutes. After pre-heating, 25 μL of freshly made 0.36 M AAPH was added to each well, and the fluorescence of the reaction mixture was measured using a Victor³ multilabel plate reader (Perkin-Elmer, Turku, Finland) once every two minutes for two hours at 37 °C, with $\lambda_{\text{Ex}} = 485$ nm and $\lambda_{\text{Em}} = 535$ nm. Sample trolox equivalents (TE) were estimated based on area-under-the-curve (AUC) method used by Ou *et al.*, (2001). Results were reported as micromoles of TE per gram freeze-dried bread sample.

Hydroxyl Radical Scavenging Capacity

The HOSC assay was conducted using fluorescein as the fluorescent probe and a Victor³ multilabel plate reader (Perkin-Elmer) according to a previously reported laboratory protocol (Moore, Yin, Yu, 2006). 30 μ L sample or solvent was reacted with 170 μ L of 9.28×10^{-8} M fluorescein, 40 μ L of 0.1990 M H₂O₂ and 60 μ L of 3.43 M FeCl₃ in microplate wells, and the fluorescence of the reaction mixture was recorded approximately once every 260 seconds for three hours at ambient temperature, with $\lambda_{\text{Ex}} = 485$ nm and $\lambda_{\text{Em}} = 535$ nm. Trolox standards and bread samples were diluted to appropriate concentrations in 50% acetone. Fresh 9.28×10^{-8} M fluorescein solution was prepared for each assay from stock solution and 75 mM sodium phosphate buffer (pH 7.4). Trolox equivalents (TE) of each sample were calculated following the same AUC method used for the ORAC TE estimation (Moore *et al.*, 2006). Results were reported as micromoles of TE per gram of freeze-dried bread samples.

DPPH• Scavenging Activity

Free radical scavenging activity was determined according to a previously reported procedure by Cheng *et al.*, (2006) utilizing stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The reaction consisted of an equal mixture of freshly prepared 100 μ M DPPH• solvent solution and grape seed extract, primed to a total volume of 200 μ L. The absorbance was measured at 515nm and compared against a standard curve consisting of six dilutions of 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) between 10 μ M to 35 μ M. The reaction was measured against the blank of solvent at room

temperature for 40 min using a Victor³ multilabel plate reader (Perkin-Elmer). Breads baked in triplicate were also measured in triplicate.

Anti-Proliferative Activity against HT-29 Cells

Anti-proliferative activity against HT-29 cells was determined according to a previously reported procedure by Wang and others (2007) and Slavin and others (2009). HT-29 human colorectal cells were cultured in cell media containing McCoy's 5A media, 10% fetal bovine serum, and 1% antibiotic/antimycotic solution. The cells were placed in a humidified incubator at 37 °C and 5% CO₂, and grown to 95% confluence, and then plated at 2,500 cells/well in a black 96-well viewplate for tissue culture. After 24 hours, the medium was replaced with 100 µL of either the control or treatment medium. The amount of cell proliferation was determined prior to treatment, and at 4, 24, 48, 72, and 96 hours after treatment using an ATP-Lite 1-step Luminescence Assay System for Perkin-Elmer. Plates were permitted to equilibrate to room temperature for a period of 30 minutes before the reading was taken. Immediately before the reading was taken on a Victor³ multilabel plate reader, 100 µL of reconstituted ATP-Lite 1step Luminescence was added to the wells. The cell media was replaced with a fresh batch of treatment or control solution every 24 hours until the plate was read.

Treatment Media Preparation. To prepare the treatment solutions, the 50% acetone extracts of the grape seed enriched and control bread were first evaporated in the nitrogen evaporator to remove acetone, and then freeze dried overnight to remove the water. Samples were then dissolved in DMSO. DMSO solutions were mixed with normal growth media to reach a concentration of 10 mg bread equivalents/mL for each sample,

and passed through a 0.2 µm retrograde cellulose syringe filters. Cells were treated with bread extracts cooked at varying times and temperatures, as well as with an unbaked control.

Statistical Analysis

Each assay was conducted in triplicate, with data reported in tables and figures as mean ± standard deviation. Differences in means between samples were determined using one-way ANOVA and Tukey's post hoc analysis. Statistical analysis was performed using SPSS software for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.05$.

Results and Discussion

Lutein and α -Tocopherol Composition

The bread samples were analyzed for their α -tocopherol and lutein contents. The tocopherol and lutein contents of the bread samples are summarized in **Figure 4.1**. The control bread samples contained higher α -tocopherol and lutein levels than the grape seed bread samples. This result may be due to the canola oil used in the control bread samples. Canola oil has been shown to have high levels of tocopherols, with α -tocopherol averaging 274.5 µg/g of oil in comparison to the 56.7 µg/g found in Chardonnay grape seed oil (Al-Saquer *et al.*, 2003). Previous studies have also found canola oil to have high lutein levels (Farre *et al.*, 2010).

In addition, it is important to note that the standard deviation for the data was fairly high. This result is possible given the triplicate samples of bread were baked by hand,

which can lead to high variability in the data. According to the statistical analysis, the grape seed bread samples did not show significantly different tocopherol and lutein levels when compared to the control samples.

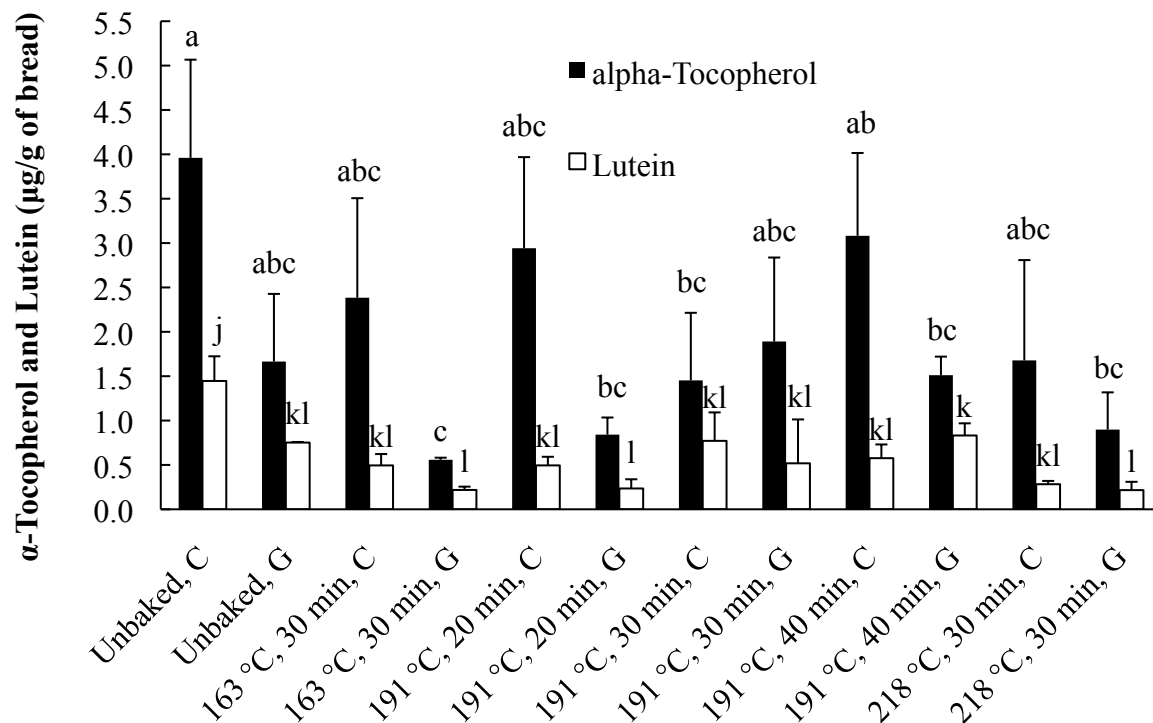


Figure 4. 1

Lutein and α -Tocopherol Content of Control and Grape Seed Breads Based on Baking Conditions.

Lutein and α -tocopherol in bread model baked at various times and temperatures compared to unbaked control bread. Solid columns represent α -tocopherol and open columns represent lutein. C indicates control bread with wheat and bread flour only. G indicates grape seed enhanced bread. The amount of α -tocopherol is consistently higher than the amount of lutein under varying baking conditions. Additionally, the amount of α -tocopherol and lutein both vary depending on time and temperature. Data marked by different letters indicate significant differences ($P \leq 0.05$), with a-c reflect the difference between tocopherol level, whereas j-l indicate the difference in lutein contents.

Total Phenolic Content

The TPC values for grape seed and control bread at each baking condition are shown in **Figure 4.2**. Grape seed bread, baked and unbaked, had significantly higher levels of phenolics than control bread. Varying baking conditions had no statistically significant effect on phenolics in control bread. Grape seed bread baked at 163 °C for 30 minutes had the highest level of phenolics with a TPC value of 25.8 mg gallic acid equivalent (GAE)/g bread and showed a statistically significant difference from breads baked at 191 °C for 20 and 40 minutes and at 218 °C for 30 minutes ($P < 0.05$). Grape seed bread baked at 191 °C for 40 minutes exhibited the lowest TPC value at 18.6 mg GAE/g bread and had a statistically significant difference from unbaked grape seed bread, bread baked at 163 °C for 30 minutes and 191 °C for 30 minutes (**Figure 4.2**).

These results suggested that higher temperature may negatively affect the level of phenolics present in the grape seed bread, with longer baking time showing a slightly greater negative influence on TPC values, but this difference is not statistically significant from the highest temperature. The large standard deviations, especially in grape seed bread baked at 163 °C and 191 °C for 30 minutes, were likely a result of variation in specific loaves of bread during the baking process. All loaves were not baked simultaneously and were not freeze-dried together.

Grape seed enrichment has been found to increase the overall antioxidant activity of bread (Peng *et al.*, 2010). Baking conditions have not been shown to significantly affect the total phenolic content of whole wheat pizza crust (Moore *et al.*, 2009). However, the total phenolic content of grape seed extracts exposed to heat treatment has

been found to increase with moderate cooking time and temperature, and decrease with heat high cooking time and temperature (Kim *et al.*, 2006). These trends found in studies on grape seed extracts correlate with the trend founds when testing the grape seed bread in the current study.

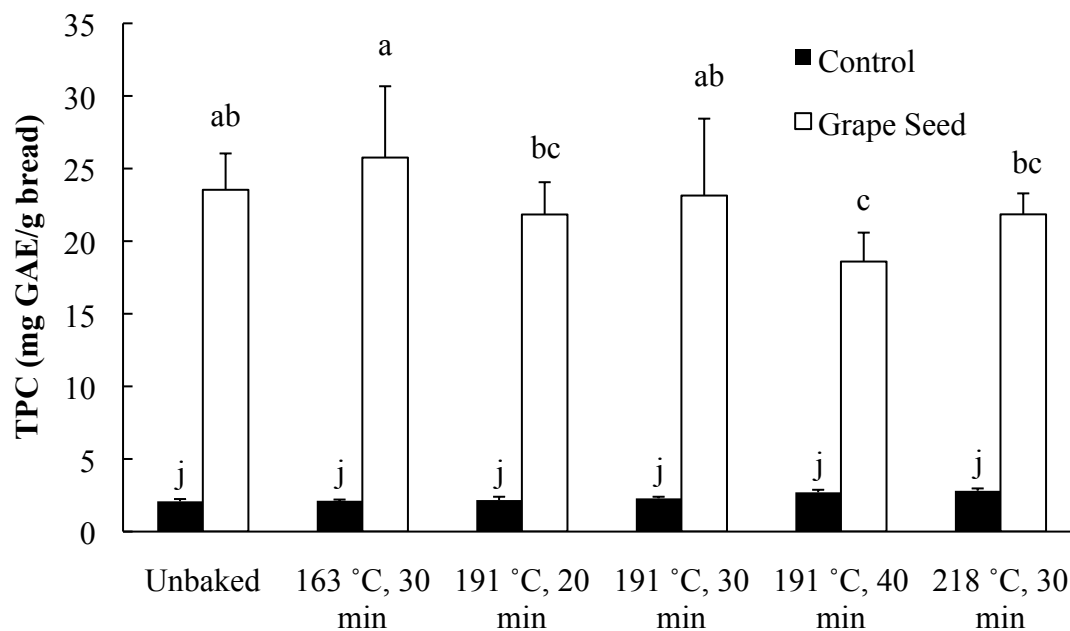


Figure 4. 2

Total Phenolic Contents of Grape Seed and Control Breads by Baking Conditions.

The total phenolic content was determined spectrophotometrically using Folin-Ciocalteu reagent. Gallic acid was used as the standard. GAE mg/g is the mg of gallic acid equivalents per g bread. Grape seed bread baked at 163 °C for 30 minutes had the highest total phenolic content, while bread baked at 191 °C for 40 minutes demonstrated relatively low phenolic content. Solid columns represent wheat bread controls and open columns represent grape seed bread. Data marked by different letters indicate significant differences ($P \leq 0.05$).

Antioxidant Properties

Supplementing whole wheat bread with Chardonnay seed flour was found to significantly increase its scavenging capacities against peroxy (ORAC) and hydroxyl (HOSC) radicals ($P \leq 0.05$). Antioxidant capacity was increased by over 1000% in some of the bread samples with the substitution of grape seed flour for a portion of the wheat flour. In addition, baking time and temperature was shown to significantly affect ORAC and HOSC values for bread samples containing grape seed flour, but did not significantly change the antioxidant activities of non-supplemented bread samples ($P \leq 0.05$) (**Figures 4.3-4.4**). The highest ORAC value of 628.6 $\mu\text{mol TE/g}$ dried sample was measured for the grape seed containing bread baked at 163 °C for 30 min (**Figure 4.3**). In contrast, the highest HOSC value of 434.1 $\mu\text{mol TE/g}$ dried sample was observed for the grape seed supplemented bread baked at 191 °C for 30 min (**Figure 4.4**). Overall, both radical scavenging capacity assays found antioxidant capacity of the bread increased by moderately lengthening the baking time, but extended baking times reduced the measured level of antioxidant capacity, although the difference in the ORAC assay was not statistically significant.

The experimental results suggested that bread supplemented with grape seed flour and oil had high potential application for developing a functional food rich in natural antioxidants. In 2004, Wu *et al.* measured ORAC of common foods consumed in the United States. Grain and cereal products exhibited radical scavenging capacities of 10-20 $\mu\text{mol TE/g}$ fresh weight, consistent with that found in the control bread samples in this study (25-38 $\mu\text{mol TE/g}$ freeze-dried weight). Foods that contained particularly high

levels of radical scavenging capacities, such as blueberries, artichokes, kidney beans, and pecans, all exhibited ORAC values between 100-200 $\mu\text{mol TE/g}$ fresh weight (Wu *et al.*, 2004). The grape seed bread exhibited ORAC values between 410-630 $\mu\text{mol TE/g}$ freeze-dried weight. Even though values reported in the current study were per freeze-dried sample weight, as opposed to fresh-weight basis, the determined ORAC values are still congruent with added health beneficial properties of grape seed bread. HOSC values showed similar support of high antioxidant levels in grape seed supplemented bread. In comparison to the values measured in this study, 21-35 $\mu\text{mol TE/g}$ of control bread, Moore and others found HOSC values for whole soft wheat and hard wheat bran to be 38.78 and 74.91 $\mu\text{mol TE/g}$, respectively (Moore *et al.*, 2006).

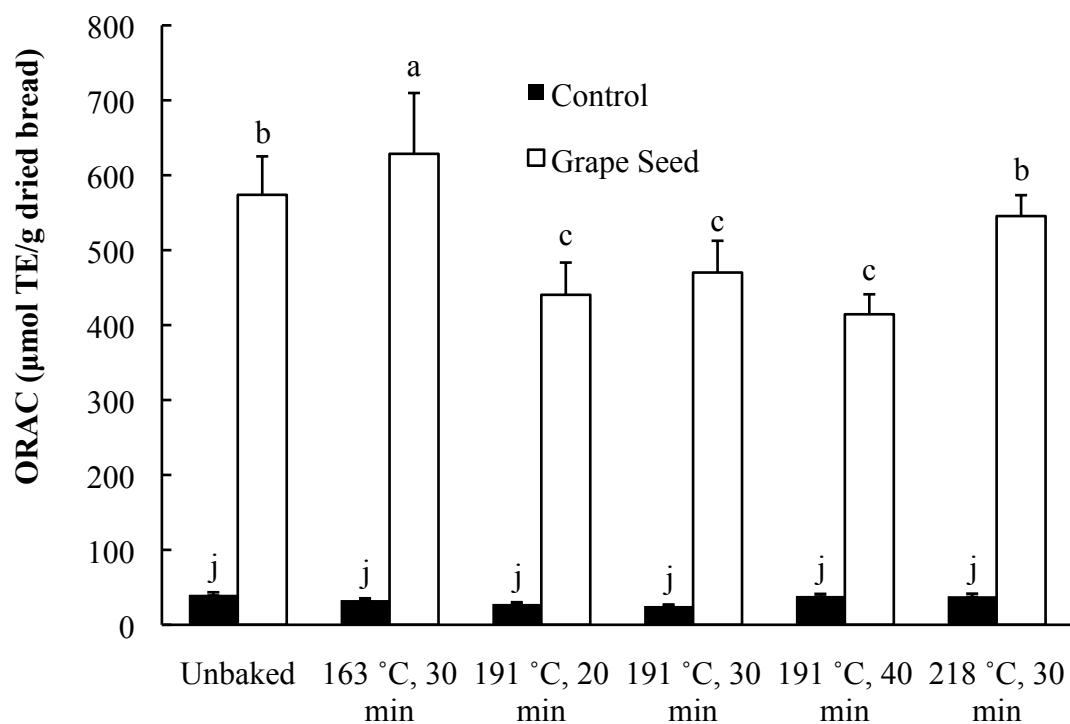


Figure 4. 3

Oxygen Radical Absorbance Capacity of Bread Extracts. Values were measured against a trolox standard and expressed as μmol of trolox equivalents (TE) per g of defatted seed flour. Mean + SD values are shown ($n = 3$). Solid columns represent wheat bread controls and the open columns represent grape seed bread. Values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$).

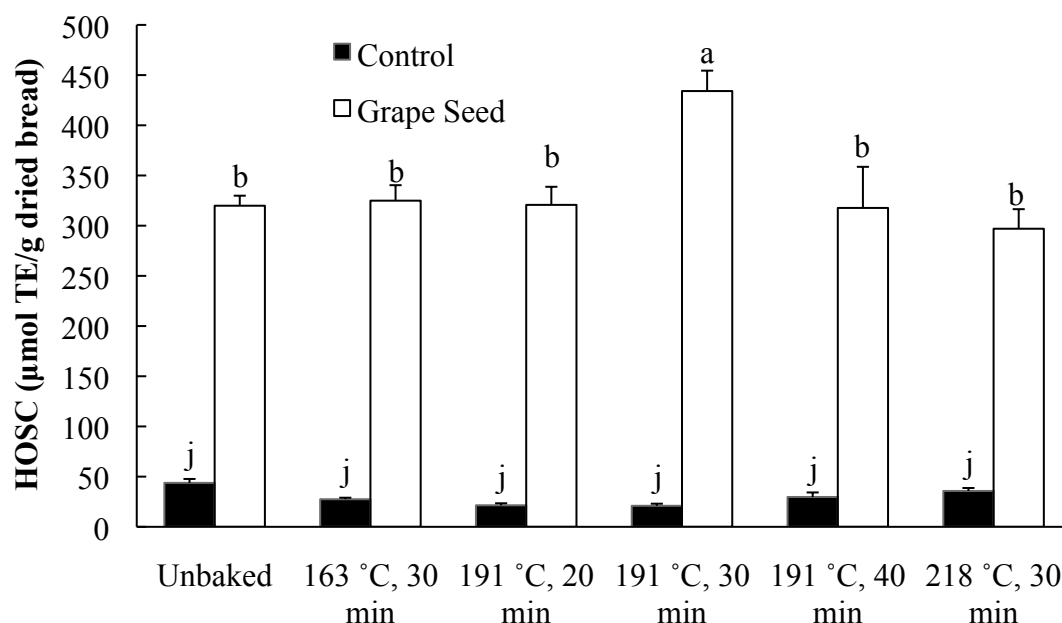


Figure 4. 4

Hydroxyl radical scavenging capacity of bread extracts. Values were measured against a trolox standard and expressed as μmol of trolox equivalents (TE) per g of defatted seed flour. Mean + SD values are shown ($n = 3$). Solid columns represent wheat bread controls and open columns represent grape seed bread. For each solvent listed, values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$)

All bread samples exhibited DPPH radical scavenging activity (RSC). All control breads exhibited significantly lower RSC ranging from 0.03 mmol TE/ g of bread to 0.101 mmol TE/g of bread as shown in **Figure 4.5**. Bread containing grape seed flour baked at 163 °C for 30 minutes exhibited the greatest RSC at 2.073 mmol TE/g bread, a value that was significantly higher than all other breads ($P \leq 0.05$). Grape seed bread baked at 191 °C for 20, 30 and 40 minutes and at 218 °C for 30 minutes were not statistically different from each other comparable with respective RSC values of 1.517, 1.107, 1.395 and 1.544 mmol TE/g of bread. Additionally the grape seed flour breads baked at 191 °C for 30 and

40 minutes showed similar RSC to the unbaked bread. The heat treatment during the process of baking appeared to have released additional antioxidants, especially at the lowest temperature combination of baking at 163 °C for 30 minutes. It was possible that baking at higher temperatures and longer times subsequently destroyed the antioxidant components initially released. RSC values of 2.4 $\mu\text{mol TE/g}$ of bread and 2.1 $\mu\text{mol TE/g}$ of bread on a per dry weight basis were previously reported for wheat bread (Michalska *et al.*, 2007; Delgado-Andrade *et al.*, 2010). The control wheat bread in the current study was ten times higher than these previously reported values. The use of different varieties, the specific use of soft wheat flour as opposed to hard wheat, as well as the use of whole wheat in the current study are likely contributors to the observed difference in RSC. Some additives that have been utilized to enrich bread include bran and rye. The RSC of wheat bread enriched with bran (which more closely approximates the whole wheat flour used here) was still lower than the current data at a reported 3.2 $\mu\text{mol TE/g}$ of bread. This was still almost 10 times lower than the control bread and 500 times less than the grape seed bread reported here (Delgado-Andrade *et al.*, 2010). Rye bread also exhibited a similar RSC of 4.9 $\mu\text{mol TE/g}$ bread (Michalska *et al.*, 2007). Overall, bread made with grape seed flour and soft whole wheat contained significant levels of antioxidants, which implied it might have potential as a functional food for augmenting daily antioxidant intake.

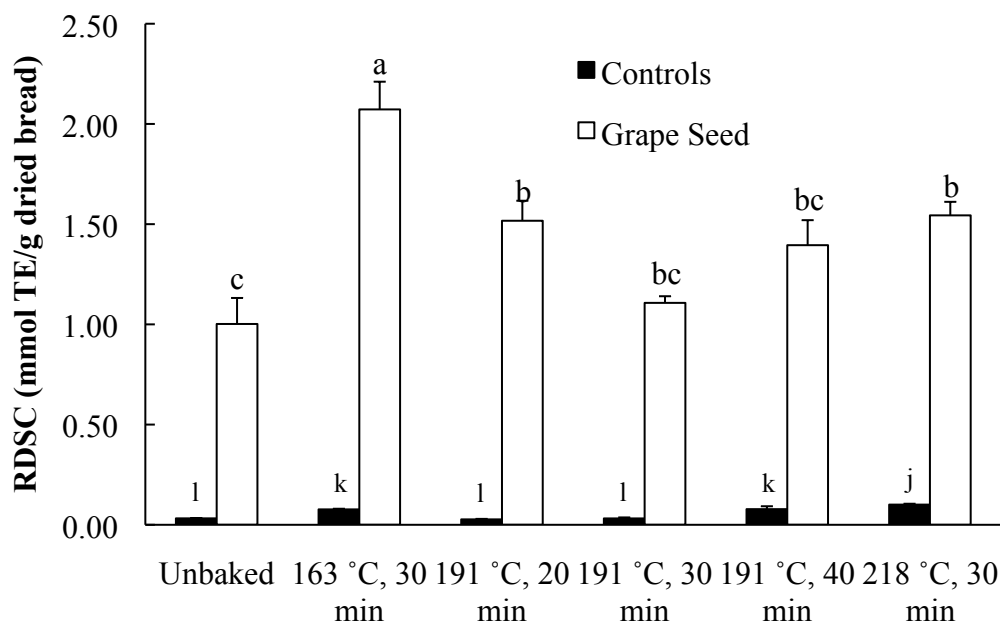


Figure 4. 5

DPPH Radical Scavenging Capacity of Bread Extracts. Solid columns represent control bread, and the open columns represent grape seed bread. Samples were evaluated for direct radical scavenging capacity (RSC) of DPPH radicals and compared to a trolox standard. Results are shown as mean \pm SD in units of mmol of trolox equivalents (TE) per gram of defatted bread sample. Columns in a series marked by the same letter are not statistically different ($n = 3$; $P \leq 0.05$).

Anti-Proliferative Activity against HT-29 Cells

The results of the anti-proliferation HT-29 cell study showed that the treatments with grape seed bread extract had significant suppressed cell proliferation. While bread samples that were baked at different times and temperatures showed slightly, but not statistically significantly different results anti-proliferative activities. Anti-proliferative activity of the bread extracts did not seem to be dependent on time and temperature of baking.

The proliferation of cells with treatments of varying baking temperatures is shown in **Figure 4.6**, which displays the effects of breads baked at various temperatures for 30

minutes. All of the grape seed containing samples, including the unbaked bread dough sample, showed significant anti-proliferative activity as compared to the vehicle control. Slight differences in cell proliferation were seen across time and temperature combinations of the grape seed bread extracts. However, the differences between the grape seed bread treatments were not statistically significant. All grape seed bread treatments showed statistically significant anti-proliferative activity when compared to the vehicle at 96 h.

The proliferation of cells with treatments of varying baking times is shown in **Figure 4.7**, which shows the effects of breads baked at 191 °C for varying lengths of time. The grape seed containing samples again significantly depressed the cell growth, as can be seen by comparing the cell growth of these samples to the cell growth of the vehicle. Again, differences were seen in the relative number of cells surviving after treatment; however, the differences between the grape seed bread treatments were not statistically significant. The grape seed treatments' inhibition of cell growth was statistically significant when compared to the vehicle treatment at 96 hours.

The anti-proliferative activity of baked and unbaked grape seed bread treatments is compared to that of baked and unbaked control bread in **Figure 4.8**. The growth displayed by cells treated with control bread extracts baked at 191 °C for 30 min was very high, exceeding the growth curve of the vehicle treatment, which had only DMSO and media. The unbaked control bread behaved oddly, showing activity roughly similar to the vehicle or slightly less until the 96 hr reading; when the amount of cells dropped significantly so that the luminescence reading was lower than that of the other samples compared here. The baked grape seed sample, which was also baked at 191 °C for 30 min, showed

antiproliferative activity and was significantly different when compared to the baked and unbaked control bread samples and the vehicle. The unbaked grape seed break extract seemed to inhibit the cell growth slightly stronger than the baked grape seed sample, although the difference is not significant. These results indicated the grape seed contained active components inhibiting the cell growth, rather than other components in the bread.

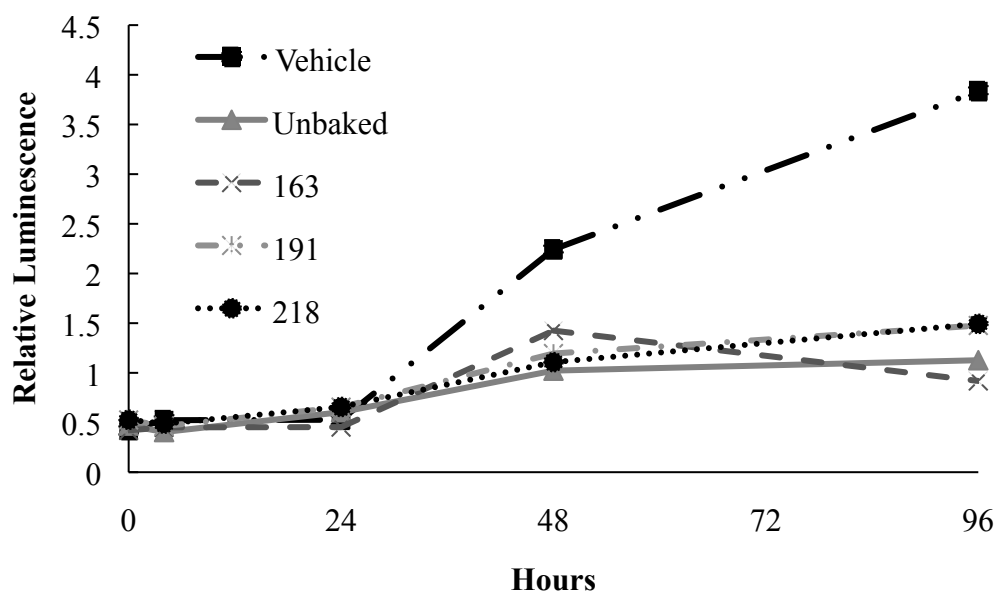


Figure 4. 6

Anti-Proliferative Effects of Grape Seed Bread Extracts by Baking Temperatures.

Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean values shown (n = 3). All samples were baked for 30 minutes. “X” with grey dash line represents the sample baked at 163 °C, “X” with dashed and one dot line represents the sample baked at 191 °C, circle with dashed line represents the sample baked at 218 °C, triangle with solid line represent the unbaked sample, and the square with dash and two dot line represents the vehicle

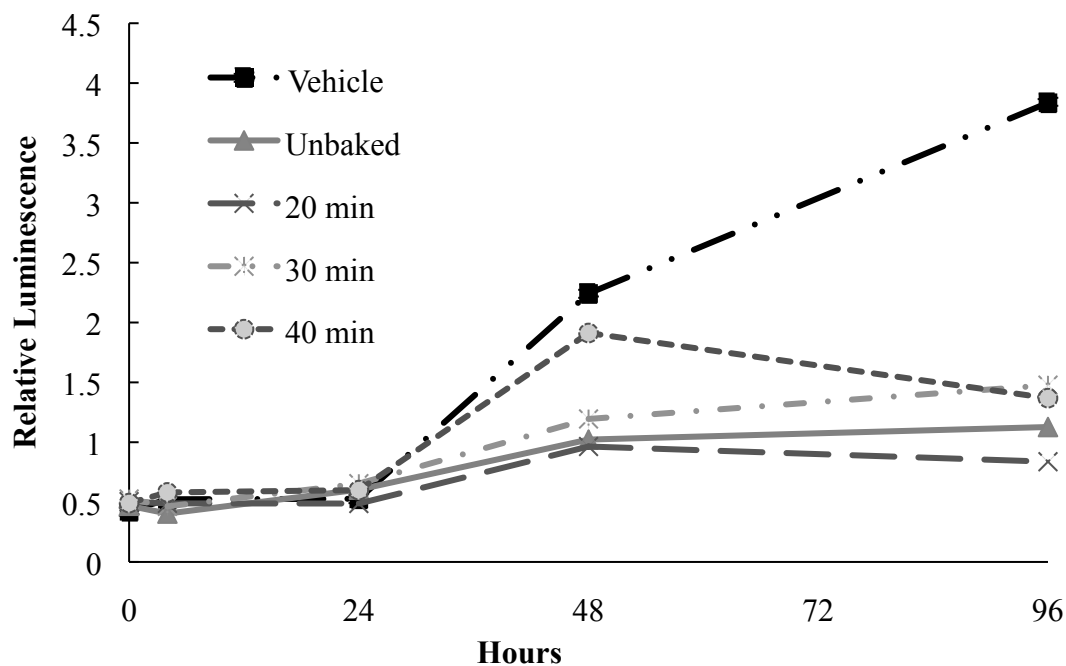


Figure 4. 7

Anti-Proliferative Effects of Grape Seed Bread Extracts by Baking Times.

Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean values shown (n = 3). All samples were baked for 163 °C minutes except for the unbaked control. “X” with dashed line represents the sample baked at 20 minutes, “X” with dash and one dot line represents the sample baked at 30 minutes, circle with dashed line represents the sample baked at 40 minutes, triangle with solid line represents the unbaked sample, and the square with dash and two dot line represents the vehicle.

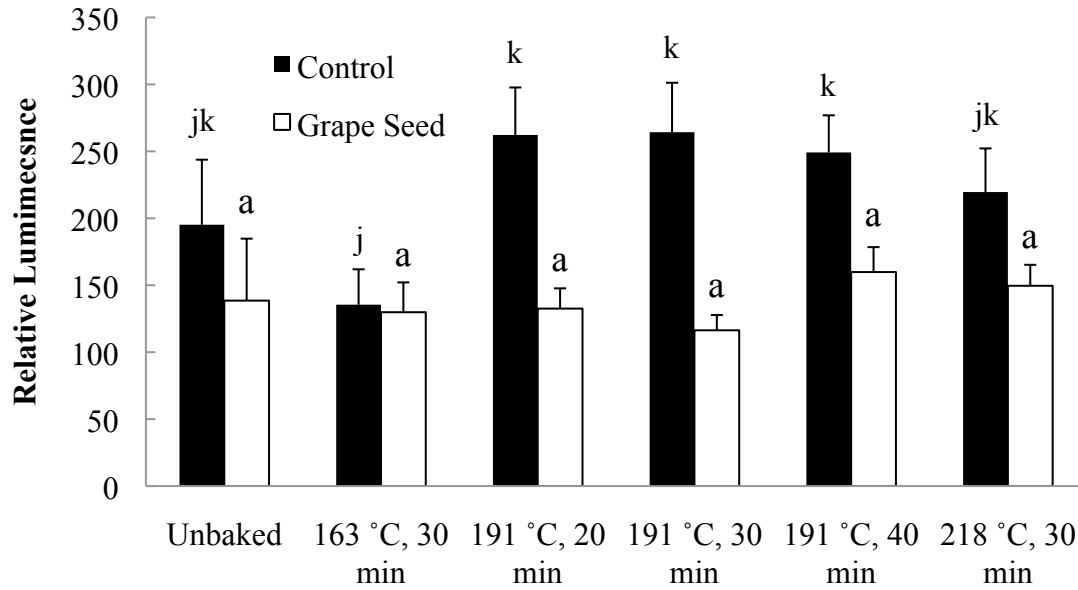


Figure 4. 8

Proliferation of Cells With Grape Seed Bread and Control Bread Treatments at 48 Hours. Relative luminescence is proportionate to the number of viable cancer cells. Solid columns represent wheat bread controls and open columns represent grape seed bread. Values marked with the same letter do not differ significantly ($n = 3$; $P \leq 0.05$).

Conclusion

In conclusion, this study demonstrated that grape seeds may be incorporated into a functional food model. The grape seed containing bread showed stronger antioxidant activity and greater amounts of health beneficial compounds than did the bread containing solely wheat. Additionally, the time and temperature of the baking process may affect the levels of antioxidant activity and health beneficial components in the finished grape seed containing bread. However, the differences seen between time and temperature combinations were small compared to the differences seen between the grape seed bread and the control (wheat only) bread. By incorporating health beneficial properties , such as those from grape seeds, into bread, there may be potential to increase the amount of antioxidants consumed by the population. Further research is necessary to determine consumer acceptance and willingness to purchase such a product.

Chapter 5: Consumer Acceptability of Functional Bread Containing Cold-pressed Grape Seed Oil and Flour

Introduction

Functional food products are defined as having health beneficial properties beyond basic nutrition (International Food Information Council, 2002). Interest in functional foods has grown in the past decade as a result of higher health care costs, and growing evidence indicates that diet is directly related to the development of chronic human diseases (Milner, 2000). Since functional food products have the potential to improve health while reducing the incidence of disease, they have become an attractive mainstay in the consumer market. Functional food and beverage sales in the US were \$24.8 billion in 2006, with sales estimated to increase by 56% to \$38.8 billion in 2011 (Zawistowski, 2010).

In this study, grape seed oils and flours were incorporated into bread to create a functional food product. Grape seeds have been shown to have many health-beneficial properties, most notably their antioxidant properties. Antioxidants can minimize the effects of oxidative stress by eliminating reactive oxidative species in the body (Limasset *et al.*, 1993). This elimination is desirable because increased oxidative stress has been linked to a higher incidence of chronic illnesses, including heart disease and cancer.

Despite the health benefits of antioxidants, research indicates that many Americans do not consume enough fruits and vegetables—valuable sources of antioxidant

components—in their diet. In 2009, the Centers for Disease Control and Prevention reported that only 32.5% of adults consumed the recommended fruit intake of two or more fruits daily, and only 26.3% of adults consumed the recommended vegetable intake of three or more vegetables daily (Grimm *et al.*, 2010). Since fruits and vegetables can be rich sources of antioxidants, these numbers suggest that many Americans may not be receiving an adequate amount of antioxidants in their diet.

Bread was chosen as a functional food model because it is a widely-consumed and highly accessible food product that could potentially increase antioxidant consumption for a large American population. However, it was still uncertain whether grape seed bread would be accepted in a consumer market, since health beneficial properties alone may not be enough to determine the consumer acceptability of a functional food. Previous research indicated that consumer acceptability of new functional food products is based on both their awareness of health benefits and favorable sensory qualities (Verbeke, 2006).

Grape seed bread is a novel functional food model, as such little previous work has been done to understand the consumer response to it. Therefore, the purpose of this study was to measure potential consumer acceptance of a functional food product containing grape seed flour and oil. Specifically, sensory evaluation was conducted by freshman university students to determine whether there would be significant preferences towards the taste, texture, and color of control bread versus the grape seed bread.

Methods

Sample Preparation

All samples were prepared and baked one to two days before the sensory analysis was conducted. First, the “wet” ingredients were mixed; 118 g lukewarm water at approximately 27 °C was added to 4 g sugar and 6 g yeast. This mixture was allowed to sit for ten minutes to proof the yeast. Meanwhile, the dry ingredients were mixed separately: 4 g salt, 33 g sugar, and 88 g wheat flour. For the control bread, 88 g bread flour was also added to this mixture. For the grape seed product, 44 g bread flour and 44 g grape seed flour was used. This “dry” mixture was then placed in a standing mixer and mixed on medium.

After the dry ingredients had been thoroughly mixed, the wet ingredient mixture was added, continuing to mix on medium speed. Additionally, 8 g oil was added to the dough; for the control sample, this was canola oil, and for the grape seed sample, grape seed oil was added. These ingredients were mixed for approximately 3-5 minutes, or until the dough had an even consistency throughout.

The dough was then removed from the mixer and placed on a cutting board lightly coated with whole wheat flour. The dough was kneaded by hand for 1-2 minutes, and then placed in a lightly oiled bowl; oil used for the bowl was congruent with the type used in the bread. The bowl was then covered with a dampened paper towel and placed into a warm oven. After allowing the dough to rise for one hour, the bowl was taken out of the oven and the dough was punched down. Then, the bowl with the dough was returned to the warm oven to rise for another 30 minutes.

Once the rising stage was complete, the dough was removed from the warming oven and transferred into bread pans for baking. The bread dough was then placed in a preheated oven at a temperature of 191 °C. The bread was baked for a total of 30 minutes; after the bread had been in the oven for 25 minutes, the top of the bread was sprayed lightly with water, and then returned to the oven for the remaining 5 minutes of baking.

The baked bread was removed from the oven after 30 minutes of baking and placed on a cooling rack for 10-20 minutes. The bread was then covered in plastic wrap and stored at room temperature for sensory analysis the following day.

Sensory Analysis

Sensory analysis was conducted in GEMS100 Freshman Honors Colloquium: Introduction to Gemstone classes at the University of Maryland, College Park. Subjects were, therefore, freshman in the Gemstone Program ($n = 78$). All trials were conducted in the same room. Tasting stalls were created using blank poster board displays to block the view of adjacent participants. For each stall, a small cup of water and soda crackers were provided, as well as a paper plate with two different bread samples, labeled A and B. The grape seed bread sample was labeled A, and the control bread sample was labeled B. The physical setup for the sensory analysis is shown in **Figure 5.1**.

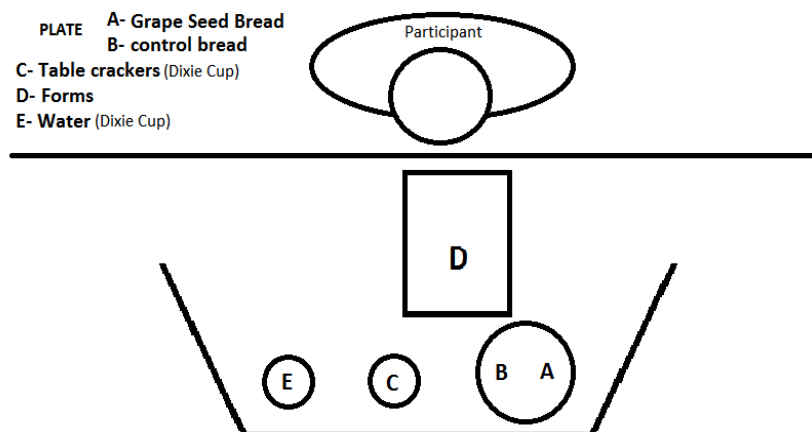


Figure 5. 1
Diagram of the sensory analysis setup.

Each class of approximately 8-10 students came separately to the testing room. After students sat down at their tasting booth, a script of instructions was read to them (see **Appendix A**). The instructions described the nature of the experiment and directed them to carefully read the consent form, which was approved by the University of Maryland Institutional Review Board (see **Appendix B**) to decide if they wanted to participate in the study. Once participants read and completed the form, they were instructed to taste both breads, cleaning their palate with the soda crackers and water between each sample. The participants were also instructed to fill out a survey (see **Appendix C**), stating their preference between the samples, and rating each sample on color, texture, and flavor. The participants were not told which bread sample contained grape seed ingredients. The favorability rating was measured on a horizontal line of length 10.3 cm, on which participants could mark their preference with a single vertical line; a mark at the beginning

of the line, at 0 cm, indicated a strong dislike for the sample, and a mark at the end of the line, at 10.3 cm, indicated a strong like for the sample. There was also a small section for comments about their sample preference. At the conclusion of the study, the surveys were collected, and a brief explanation of the study was provided.

Data Analysis

The preferences indicated on each survey were consolidated; the markings of the participants were measured so a numerical value could be assigned to each response. This number was the length measurement from the left end of the line on the survey in centimeters. Again, a small number corresponds to disfavor while a larger number corresponds to favor. Lastly, the values from all the surveys for each individual question were averaged and the standard deviation was determined. Statistical analysis was performed on the mean preference ratings for color, texture, and taste for the grape seed and control breads. The test run was a paired 2-tailed Student's T-test, with pairing between the responses made by each student. Differences in means between samples were determined using one-way ANOVA. Statistical analysis was performed using SPSS software for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.001$.

Results

In this study, there were 78 participants, all freshman college students at the University of Maryland, College Park. Of the 78 participants, 11 had no preference between the two samples, 53 preferred the control bread, and 14 preferred the grape seed bread.

The results for the mean preference ratings for taste, texture, and color, as well as their standard deviations, can be found in **Table 5.1**. These results indicate that participants preferred the color, texture, and flavor of the control over the grape seed bread. The responses had large relative variations, with %CV of 35.5-44.7 % for grape seed bread, and 23.5-26.5 % for control bread, for means between values of 5-10. However, because of the large sample size, the difference in means between grape seed bread and control bread were found to be significantly different in all three categories of color, texture, and flavor ($P < 0.001$).

While the majority of participants ranked the sensory properties of the control bread higher than that of the grape seed bread, overall both samples were acceptable, as measured by the mean preference values for each sample category. A value of 5.15 out of the 10.3 scale would indicate neutral favorability, while any values lower than 5.15 show disfavor. The distribution of expected mean preference values were estimated by calculation 95% confidence intervals for each sample, and in each case, was found to be higher than 5.15 (See **Table 5.2**). This indicates that in all subsequent sensory evaluations, the mean of the preference value for both grape seed and control bread in

each category of color, texture, and flavor would be expected to fall above neutral preference.

To analyze the distribution of the data further, a box and whisker plot were created and presented in **Figure 5.2**. When comparing the lower quartile limit of the control sample to the median value of the grape seed bread for each sensory category of color, texture, and flavor, it can be seen that the control sample lower quartile limit increases relative to the grape seed bread median from texture, to color, to flavor. This suggests that among those surveyed, the preference for control over grape seed bread was the strongest in the case of texture, and weakest in the case of flavor. Additionally, there are relatively few outliers in the box and whisker plot, showing that the mean values were not significantly skewed by the presence of outliers.

Table 5. 1

Mean Preference Ratings of Color, Texture, and Flavor for Grape Seed and Control Breads with %CV.

	Color	Texture	Flavor
Grape Seed Bread	5.7 ± 2.0 (35.5%)	5.5 ± 2.4 (44.7%)	6.4 ± 2.5 (38.5%)
Control Bread	7.1 ± 1.7 (23.5%)	7.7 ± 2.0 (26.5%)	7.8 ± 2.0 (25.2%)

Each rating is out of 10.3, with higher ratings showing more favorability. Grape seed and control breads were statistically different (n=78, $P=0.001$).

Table 5. 2

Color, Texture, and Flavor Mean Preference Interval at 95% Confidence Level for Grape Seed and Control Breads.

	Color	Texture	Flavor
Grape Seed Bread	5.2-6.1	4.9-6.0	5.8-6.9
Control Bread	6.7-7.5	7.2-8.1	7.3-8.2

Each rating is out of 10.3, with higher rating showing more favorability.

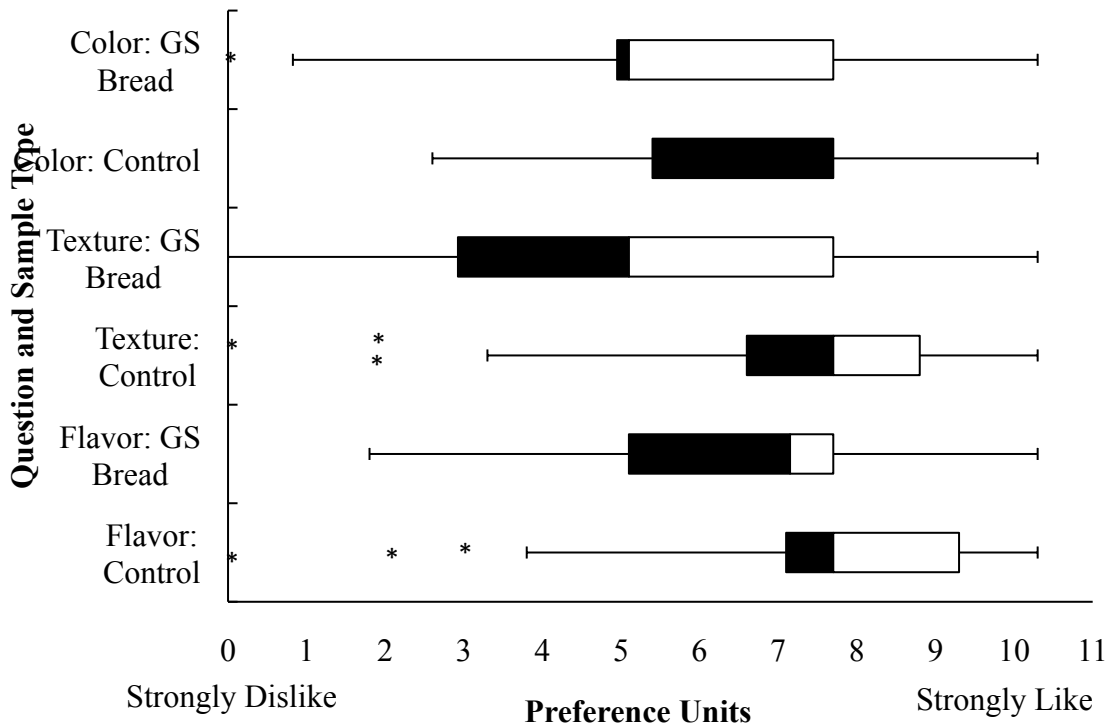


Figure 5. 2

Box and Whisker Plot of Grape Seed Bread Sensory Analysis Data. The box indicates the interquartile range. Outliers are marked with asterisks. Outliers are calculated by determining if they were below $Q_1 - 1.5 \times IQR$ or above $Q_3 + 1.5 \times IQR$. There were 78 participants. Preferences were measured from a 0 to 10.3 cm scale.

Conclusion

The results indicated significant differences between consumer acceptability of the control bread and the grape seed bread. More respondents preferred the sensory qualities of the control bread than the grape seed bread, with the most prominent difference noted in the textures of the two breads. Of the three categories evaluated, both the control and the grape seed bread received their highest rating in flavor. The difference in preference between the two breads was much smaller for flavor than the difference between the two

bread for texture. Despite the preference for control bread over grape seed bread, many of the respondents did indicate that they had an overall favorable perception of the grape seed bread. Mean preference values show that the grape seed bread scored above a neutral rating for all three sensory categories tested. Data from this study suggest that consumers might be willing to consume a bread product containing grape seeds; however, further research is required to determine if these results would be true for a larger population.

Chapter 6: Conclusion

Three comprehensive studies were conducted to determine the feasibility of creating a functional food product rich in natural antioxidants that could be used to lower the risk of chronic illnesses in the general population. Grape seeds were selected as the source of nutraceuticals, both for their high antioxidant activity and their current role as an agricultural by-product. Bread was chosen as the functional food model because it is commonly consumed by most socio-economic classes in the United States. In addition, the baking conditions for bread are easily modified to test the effect of processing on the chemical composition and health beneficial properties of the functional food. Finally, a sensory survey was used to evaluate the acceptability of the proposed functional food, and to determine if the addition of grape seed flour and oil significantly affected the appeal of bread to consumers.

The first study quantified the health beneficial properties of seeds from five different grape varieties: Chardonnay, Concord, Norton, Ruby Red, and White. Extractions were made from cold-pressed grape seed flour and oil samples using four different solvents. Grape seed flours and oils were found to contain varying levels of antioxidant properties, chemical composition, and anti-proliferative activity against colon cancer cells. Of the grape varieties tested, Chardonnay exhibited the highest level of health beneficial properties, overall, and was chosen for incorporation into the bread model in the second study. In addition, 50% acetone was found to be the most effective solvent for

extracting phytochemicals from grape seeds, and thus was used for subsequent chemical assays in the second study.

The second study compared the health beneficial properties of incorporating grape seed flour and oil into a standard bread recipe with those of a control bread product. The addition of grape seed flour and oil increased the antioxidant activity of the bread by roughly 10-fold, as well as significantly improving its carotenoid and α -tocopherol contents. Antioxidant activity and chemical composition of the bread also varied with the baking time and temperature, although only grape seed bread treatments exhibited significant differences between time and temperature combinations. However, altering cooking time and temperature did not significantly change the antioxidant profile in the control bread. The decrease in antioxidants in the grape seed bread, but not in the control could potentially be attributed to less thermally stable antioxidants present in grape seeds in comparison to those found in wheat flour, although the possibility was not evaluated in detail.

The sensory evaluation conducted in the third study analyzed consumer preference for control and grape seed breads in terms of appearance, taste, and texture. Preference for control bread over grape seed bread was found to be statistically significant for all 3 sensory factors surveyed. However, both the control bread and the grape seed bread had overall positive reviews, suggesting that consumers would be willing to consume this novel functional food product. These results suggest that grape seed bread can potentially become a viable commercial functional food product; however, further research is necessary to determine if the positive impressions detected here would translate into consumer purchases and consumption, particularly when weighing the product's novel

sensory properties and possible increased cost against the available health-beneficial properties.

There is great potential value in creating a grape seed bread commercial product. The incorporation of grape seed flour and oil into the bread recipe has been shown to significantly increase its potential health beneficial properties. In addition, because grape seeds are currently a waste-product of juice and wine production, the cost to produce this functional food would be expected to be significantly lower than purchasing current “superfoods” promoted as significant sources for natural antioxidants. This product would also increase the commercial value of grape seeds, and thus benefit the agricultural community by increasing the overall value of grape production. However, before such a product could be sold to consumers, its safety and long term health effects should be evaluated.

Limitations and Future Research Directions

The main limitation and direction for further study was the lack of *in vivo* data directly supporting the health-beneficial properties that were measured for bread containing grape seed flour and oil. While extensive literature exists supporting the links between antioxidant compounds, chemical measurements for antioxidant activity, reduced oxidative stress in lab animals, promoting health in lab animals, and finally promoting health in humans, the overall chain of thought is tenuous at best, and often claimed to only apply in a case-by-case process due to the complexity of metabolism in the body. With such a study, the consumption of the grape seed enriched bread could be directly linked with reduction of biomarkers of chronic illnesses in the test subjects. However, the cost to run such an animal study or clinical trial was prohibitively expensive at this time.

A much simpler problem to tackle would be the small number of baking conditions evaluated in the second part of this study. While 5 covariate points were sufficient for individually examining the effects of baking time and temperature, more would be beneficial to study the interaction between time and temperature. Additional points would have also increased the robustness of the conclusions made regarding the effects of baking conditions on health beneficial properties of the bread. More replications of the existing 5 baking conditions would also improve the strength of the conclusions drawn from the results.

Another direction for further study existed with the consumer sensory survey. The study makes conclusions based upon the assumption that first year college students will be representative of consumers with respect to their taste and perception when evaluating a novel functional food. However, the assumption was made out of necessity, because of the infeasibility of surveying a large sample of people across the spectrum of the population, including different ages, ethnic groups, and socioeconomic classes. However, all of these factors could conceivably alter a consumer's perception of bread containing grape seeds. These factors could be accounted for if the sample size were expanded, given more materials for baking bread, and time for finding suitable subjects and conducting sensory surveys.

This study could have also evaluated other grape varieties. The grapes studied were limited by the grape seeds provided externally. However, with additional funding, more varieties could have been evaluated by purchasing grape seeds from industry and renting machinery to cold-press the seeds to obtain flour and oil samples. This would

increase the agricultural and economical value of this research, especially if the purchased grape varieties were the most commonly used by the juice and wine industry.

Finally, the feasibility of commercially producing the novel bread product developed in this project could be further studied using a thorough cost-analysis. To predict the price of this product, the cost of securing the grape seeds for the modification to a whole wheat bread recipe would have to be considered in conjunction with the challenge of modifying existing infrastructure to cold-press grape seeds on an industrial scale and adding the resulting flour and oil to current bread production lines. A survey could then be performed to assess the likelihood of consumers purchasing this bread product, given the knowledge that it could reduce the risk of chronic illnesses by supplementing diet with natural antioxidants. Toxicity and efficacy studies should also be completed on grape seed meal specifically before the idea is brought into the commercial market. Again, limitations in time and material prevented the completion of such an analysis at this time.

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Appendices

Appendix A: *Script*

[A Team IMMUNE member will enter the room that the class is meeting with prepared bread samples, small glasses of water, and table crackers. The student will introduce themselves to the class.]

I am [name], a Gemstone student from Team IMMUNE. We are conducting research on bread containing grape seed ingredients, which we have prepared. Part of this research includes evaluating consumer opinions about the grape seed bread through taste tests. Your participation in our taste test will help us to complete our team's thesis and contribute to academic literature regarding this type of food modification. Your participation is entirely voluntary, and there is no penalty for declining to participate. If you are interested in participating in our study, please thoroughly read and complete this consent form. [Hand out consent forms to students that wish to participate.] Please note that the samples you will be asked to taste as part of this study contain wheat flour, grape seed flour, grape seed oil, vegetable oil, sugar, yeast, salt, and water. Please do not participate in this study if you have an allergy to bread or any of the ingredients or have a medical condition that prohibits you from consuming gluten.

[Team IMMUNE member will wait for students to read and sign consent form if they still wish to participate, and then collect the papers and put them in a sealed envelope.]

You will be given two samples of bread. Between tasting the two different samples you should clean your palate by eating the cracker and taking a sip of water. For each sample, you will circle an answer or rate your reactions by marking with a vertical tick mark between strongly dislike and strongly like scale line. Please mark with a clear line because they will be measured for data collection. Please do not include your name or any identifying information on the survey.

Please do not converse or interact with any other participants during tasting. If you have any questions regarding this study, feel free to ask me at any time. You can choose to stop participating in this study at any time.

[Team IMMUNE member will then give each student two samples of bread. Sample A will be the control bread sample; Sample B will be the grape seed sample. Also, each participant will be given a small glass of water and table crackers. Once the participant has finished the survey, the Team IMMUNE member will collect the surveys and place them in a separate sealed envelope.]

Thank you for your time! Our Gemstone Team is in our last year of research. We have already completed a comprehensive chemical analysis of many different types

of grape seed flours and oils, and designed our own novel grape seed bread through baking many prototypes. We are now using chemical tests to assess the antioxidant properties of our bread, and are using these surveys to assess if the bread is acceptable to consumers. If you have any further questions regarding this study or our team's project, please feel free to contact me directly or e-mail our group at immune@umd.edu.

[Any remaining bread samples, table crackers, or water glasses will be thrown away. The IMMUNE member will then leave the classroom and return the sealed envelopes to Dr. Yu's office]

Initials _____ Date _____

Appendix B: *CONSENT FORM*

Project Title	Consumer Acceptance Analysis of Grape Seed Bread
Why is this research being done?	This is a research project being conducted by the Gemstone IMMUNE undergraduate research team in cooperation with the Laboratory of Nutraceuticals and Functional Foods at the University of Maryland, College Park. The purpose of this research project is to promote the adoption and utilization of grape seeds integrated into daily diet. Thus, the objective of this experiment is to examine the consumer perception of prepared bread that contains grape seed flour and oil that are rich in antioxidants.
What will I be asked to do?	The procedure involves your evaluation of a grape seed bread sample and a control wheat bread sample. You will be given bread to taste and then asked to complete a survey about the bread's taste, texture, and color. The entire procedure should take no longer than approximately twenty (20) minutes.
What about confidentiality?	<p>We will do our best to keep your personal information confidential. To help protect your confidentiality, we will not collect any identifying information on the surveys. Surveys will be kept in a locked office.</p> <p>If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland, College Park or governmental authorities if you or someone else is in danger or if we are required to do so by law.</p>
What are the risks of this research?	There may be some risks from participating in this research study. If you are allergic to any of the ingredients in the bread (wheat flour, bread flour, salt, sugar, water, yeast, oil, grape seed flour, grape seed oil), you should not participate in this study. If you have a medical condition that makes you unable to eat gluten products, you should not participate in this study. There are no other known risks associated with participating in this research project.

Initials _____ Date _____

Project Title	Consumer Acceptance Analysis of Grape Seed Bread	
What are the benefits of this research?	The benefits to you include increased awareness of the health-beneficial properties of antioxidants, specifically in grape seed derivatives.	
Do I have to be in this research? May I stop participating at any time?	Your participation in this research is completely voluntary. You may choose not to take part at all. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this study or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify.	
What if I have questions?	<p>This research is being conducted by Dr. Liangli Yu of the Department of Nutrition & Food Science at the University of Maryland, College Park. If you have any questions about the research study itself, please contact Dr. Liangli Yu at: 3303 Marie Mount Hall, College Park, MD 20740, (301)-405-0761, or lyu5@umd.edu.</p> <p>If you have questions about your rights as a research subject or wish to report a research-related injury, please contact: Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742; (e-mail) irb@deans.umd.edu; (telephone) 301-405-0678</p> <p>This research has been reviewed according to the University of Maryland, College Park IRB procedures for research involving human subjects.</p>	
Statement of Age of Subject and Consent	<p>Your signature indicates that:</p> <ul style="list-style-type: none"> you are at least 18 years of age; the research has been explained to you; your questions have been fully answered; and you freely and voluntarily choose to participate in this research project. 	
Signature and Date	NAME OF SUBJECT	
	SIGNATURE OF SUBJECT	
	DATE	

Appendix C: *Team IMMUNE Survey*

Please taste each sample, eating the provided crackers and drinking some of the water in between each sample to cleanse your palate.

1. Do you have a taste preference for one of the samples?

Yes

No

2. Which sample do you prefer? Why do you prefer this sample?

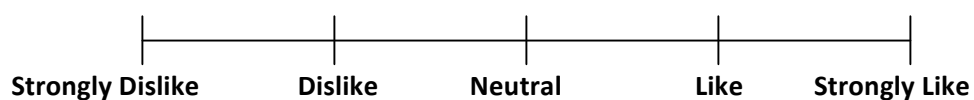
A

B

No Preference

Comments (optional):

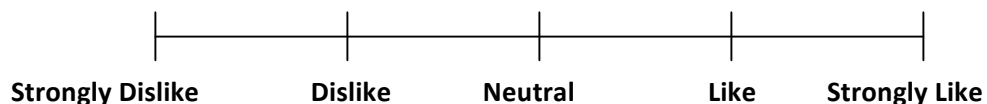
3. How much do you like the color of Sample A?



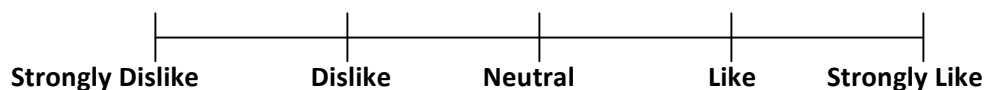
4. How much do you like the texture of Sample A?



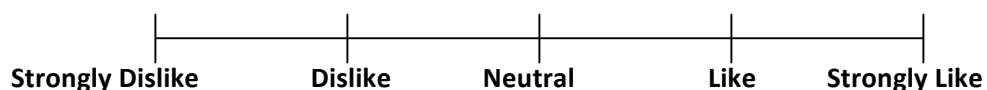
5. How much do you like the flavor of Sample A?



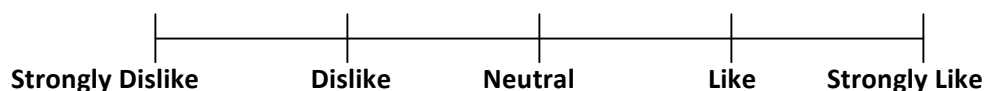
6. How much do you like the color of Sample B?



7. How much do you like the texture of Sample B?



8. How much do you like the flavor of Sample B?



Glossary

Anthocyanin: a common flavanoid which gives grapes and grape seeds their purple coloring. Anthocyanin's health benefits include anti-inflammation of blood vessels and reduction of platelet coagulability (Mazza, 2007).

Anticarcinogenic: having to do with preventing the development of cancer

Antimycotic: antifungal

Antioxidants: molecules that reduce oxidative stress in the body by slowing and preventing free-radical oxidation reactions, which occur naturally in the human body anti-proliferative activity

Atherosclerosis: the hardening of the arteries caused by formation of plaques in the arteries. It can lead to strokes and heart attacks.

[Beta]-carotene: a carotenoid that is a precursor to vitamin A. It's strongly red-orange colored and common in fruits and plants (i.e. carrots).

Bioactive: having an effect on living tissue

Bioavailability: physiological availability of a chemical in the body for use

Biomarkers: biological molecules used as an indicator to measure progress of processes

Carotenoids: photosynthetic plant pigments that are fat-soluble and possess antioxidative properties. Examples include beta-carotene and lutein.

Cold pressed: temperature controlled processing technique in which oil is produced with the use of low heat technique. High heat degrades the flavor and the nutritional value of the oil product, so cold pressing yields higher quality.

Cytotoxicity: toxicity to cells

Dietary fibers: parts of grains, fruit, and vegetables that contain cellulose. They are not digested by the body and help the intestine to absorb water.

Flavanoids: most common group of polyphenolic compounds in the human diet. They're commonly found in plants and are also known as vitamin P. Key types of flavanoids present in grapes and grape seeds are proanthocyanidins, anthocyanins, and resveratrol.

Free radicals: strong oxidizing molecules that exist in the human body as a natural product of metabolism and function

Functional food: food that provides an additional physiological benefit beyond that of its inherent nutritional value (Bidlack *et al.*, 2005)

Glutathione (GSH): tripeptide that have an peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain. It has antioxidative properties and can exist in both reduced (GSH) and oxidized (GSSG) states.

Glycation: bonding of sugar to a lipid of protein

Homeostasis: the stable and normal state

Lutein: a type of carotenoid that may reduce the risk of certain eye diseases (Landrum *et al.*, 2001).

Low-density lipoprotein (LDL): one type of carrier of cholesterol and fat in blood, linked to increased risk of heart disease

Malignant: tending to get worse or spread

Monocyte: a type of white blood cell and part of the human immune system.

Nutraceutical: a food that has specific health benefits, combines the words "nutrition" and "pharmaceutical"

Oxidative stress: imbalance between the reactive oxygen species and a biological system

to detoxify the reactive intermediates. It can be caused by overexposure to sunlight, smoking, daily exposure to certain chemicals, and improper diet.

Plaque: buildup of white blood cell deposits on the inside wall of an artery

Polyphenols: conjugated chemicals that have been shown to have antioxidative properties. They're commonly found in plants and are also known as phenoloids, phenols, or phenolics.

Relative luminescence: the amount of light emitted, corresponds with the number of living cells

Resveratrol: a type of polyphenol that is naturally produced by plants.

Saponification: chemical process by which fatty acids from tryglycerides are separated from glycerol to enable quantification by gas chromatography

Tocotrienol: a member of the vitamin E family. Vitamin E is made up of four tocopherols and four tocotrienols. Vitamin E is an important antioxidant in the body.

Unsaturated fatty acids: carboxylic acids with a long unbranched and unsaturated tail. They are unsaturated because they have one or more double bonds.

Thrombosis: formation of a blood clot within a blood vessel